Prion protein in cardiac muscle of elk (Cervus elaphus nelsoni) and white-tailed deer (Odocoileus virginianus) infected with chronic wasting disease

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To investigate the possible presence of disease-associated prion protein (PrPd) in striated muscle of chronic wasting disease (CWD)-affected cervids, samples of diaphragm, tongue, heart and three appendicular skeletal muscles from mule deer (Odocoileus hemionus), white-tailed deer (Odocoileus virginianus), elk (Cervus elaphus nelsoni) and moose (Alces alces shirasi) were examined by ELISA, Western immunoblot and immunohistochemistry (IHC). PrPd was detected in samples of heart muscle from seven of 16 CWD-infected white-tailed deer, including one free-ranging deer, and in 12 of 17 CWD-infected elk, but not in any of 13 mule deer samples, nor in the single CWD-infected moose. For white-tailed deer, PrPd was detected by Western blot at multiple sites throughout the heart; IHC results on ventricular sections of both elk and white-tailed deer showed positive staining in cardiac myocytes, but not in conduction tissues or nerve ganglia. Levels of PrPd in cardiac tissues were estimated from Western blot band intensity to be lower than levels found in brain tissue. PrPd was not detected in diaphragm, triceps brachii, semitendinosus, latissimus dorsi or tongue muscles for any of the study subjects. This is the first report of PrPd in cardiac tissue from transmissible spongiform encephalopathy-infected ruminants in the human food chain and the first demonstration by immunological assays of PrPd in any striated muscle of CWD-infected cervids.

INTRODUCTION

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE) that affects wild and captive cervids, including mule deer (Odocoileus hemionus), white-tailed deer (Odocoileus virginianus), wapiti or Rocky Mountain elk (Cervus elaphus nelsoni) and moose (Alces alces shirasi) (Williams & Young, 1992; Williams, 2005; Kreeger et al., 2006). In common with other TSEs, such as scrapie of sheep and goats, bovine spongiform encephalopathy, transmissible mink encephalopathy and the human TSEs, a diagnostic hallmark of CWD infection is accumulation of a disease-associated prion protein, PrPd, in the central nervous system (CNS). PrPd, which is an aberrantly refolded conformational isoform of a normal cellular glycoprotein, PrPC (Prusiner, 2004), replicates during infection by inducing the conversion of PrPC to the PrPd conformation. PrPd is partially protease-resistant, unlike PrPC, and this difference is the basis of many molecular detection protocols currently used; it may also account for extracellular accumulation of PrPd in vivo and survival through the digestive tract of exposed cervids.

The widespread involvement of both lymphoid and nervous-system tissues as sites of accumulation of PrPd during scrapie and CWD infection has been described extensively (Hadlow et al., 1982; van Keulen et al., 1995, 1996; Sigurdson et al., 1999, 2001; Miller & Williams, 2002; Spraker et al., 2002b), but recent investigations have also demonstrated the presence of PrPd in skeletal muscles of TSE-infected laboratory mice and hamsters, of sheep, deer and humans (Bosque et al., 2002; Bartz et al., 2003; Glatzel et al., 2003; Thomzig et al., 2003; Andréoletti et al., 2004; Mulcahy et al., 2004; Ashwath et al., 2005; Casalone et al., 2005; Angers et al., 2006; Thomzig et al., 2006). The levels of PrPd that accumulated in muscles were lower than those found in CNS or lymphoid tissue; however, in rodents, muscle-derived PrPd was shown to be infectious (Bosque et al., 2002; Angers et al., 2006). The detection of PrPd (designated PrPSc in scrapie) in skeletal muscles of scrapie-infected sheep (Andréoletti et al., 2004) and of infectivity in skeletal muscle of CWD-infected mule deer (Angers et al.,...
2006) has raised the level of concern on the issue of potential human health risks that might be encountered by consuming prion-containing meat.

Based on epidemiological evidence and transgenic mouse bioassays, CWD seems not to be transmitted to humans (Belay et al., 2004; Kong et al., 2005). However, because deer and elk are food sources, hunters in areas where CWD is known to occur are advised not to consume brain, spinal cord or lymph nodes, not to kill and eat any animals that are obviously unwell and to submit samples to state game agencies for testing (for example, http://www.wildlife.utah.gov/news/05-10/cwd.php; http://wildlife.state.co.us/CWD/index.asp; http://www.dnr.state.wi.us/org/land/wildlife/wheat/issues/CWD/index.htm; http://gf.state.wy.us/services/education/cwd/index.asp; http://www.ngpc.state.ne.us/wildlife/guides/cwd/aq.asp; http://www.sdgp.info/Wildlife/hunting/BigGame/CWD.htm). Information on PrP\textsuperscript{D} in muscle tissue of cervids is therefore of interest to hunters, as well as pertinent to research questions on the pathogenesis of CWD in its natural hosts. An extensive immunohistochemical analysis of tissue samples from naturally infected mule deer (Spraker et al., 2002a) and a report of two elk inoculated intracerebrally (i.c.) with scrapie (Hamir et al., 2004) both failed to detect PrP\textsuperscript{D} in muscle tissues. However, Angers et al. (2006) recently reported successful TSE transmission to transgenic mice inoculated i.c. with extracts of skeletal muscle from CWD-infected mule deer. We report here the detection of PrP\textsuperscript{D} in cardiac muscle of two species of cervid as a result of our investigation of striated muscle samples from CWD-infected mule deer. We thaw partially at room temperature in a biological safety cabinet and samples (approx. 200 mg) were cut from the interior of the muscle. Homogenates were prepared and processed following manufacturer’s procedures. Duplicate wells were run for each processed sample and absorbance values were measured. Calculation of the absorbance cut-off value for PrP\textsuperscript{D} detection followed procedures specified by the manufacturer for cervid retropharyngeal lymphnode assays.

**METHODS**

**ELISA.** Muscle tissues were assayed for prion protein resistant to digestion by proteinase K (PK) (PrP\textsuperscript{res}) by using chronic wasting disease antigen test kits (Bio-Rad). Frozen muscle tissue was allowed to thaw partially at room temperature in a biological safety cabinet and samples (approx. 200 mg) were cut from the interior of the muscle mass. Care was taken to minimize potential contamination of the sample with the surface of the muscle. Homogenates were prepared and processed following manufacturer’s procedures. Duplicate wells were run for each processed sample and absorbance results \((A_{450}-A_{620})\) for the two wells were averaged. Calculation of the absorbance cut-off value for PrP\textsuperscript{D} detection followed procedures specified by the manufacturer for cervid retropharyngeal lymphnode assays.

**Sample preparation, gel electrophoresis, Western blot transfer and development.** To prepare samples for gel electrophoresis and subsequent Western blot analysis, tissue homogenates (10%, w/v) from frozen tissues were prepared in 0·1 M Tris/HCl (pH 8·0), 0·1 M NaCl (TBS100). Frozen tissue was allowed to thaw partially at room temperature in a biological safety cabinet and samples (approx. 150–200 mg) were cut from the interior of the muscle mass as described above for ELISA samples. Homogenates were prepared in 1·5 ml TBS100 by processing in FastPrep vials (Q-Biogene) containing about 0·25 ml 1·0 m zirconia/silica beads (BioSpec) and one 0·25 inch ceramic bead (Q-Biogene) in a FastPrep FP120 bead mill homogenizer (ThermoSavant) for two cycles of 30 s each on high speed (setting 6·5), with 5 min room temperature cooling between cycles. Homogenates were stored in the bead vials at \(-20^\circ\text{C}\) and reprocessed for 10 s at speed 4 in a FastPrep to resuspend tissue particles aggregated by freezing. Tissue slurries were removed from large remaining particles of connective tissue with the aid of a fine-tip transfer pipette (Samco Scientific). Aliquots (0·1 ml) were treated with 100 μg PK ml\textsuperscript{-1} (InVitrogen) and 2 units DNase (Benzonase Nuclease; Novagen) in TBS100, 0·5% each of Nonidet P-40 (Pierce) and sodium deoxycholate, 1% sodium N-lauroylsarcosine (SLS), 1 mM each of CaCl\textsubscript{2} and MgCl\textsubscript{2} for 30 min at 45°C. Digestion was terminated by addition of protease inhibitor (Pefabloc; Fisher Scientific) to 4 mM. Samples were centrifuged at 1200 g for 3 min and supernatants, after transfer to 1 ml microfuge tubes, were made up to 2% in SLS and prewarmed to 37°C. Methods for sodium phosphotungstic acid (NaPTA) precipitation were adapted from published protocols (Safar et al., 1998; Wadsworth et al., 2001; McCutcheon et al., 2005). A 1/12 volume of 4% (w/v) NaPTA, 0·17 M MgCl\textsubscript{2} adjusted to pH 7·4 with NaOH, was added at 37°C and samples were incubated for 30 min with shaking at 37°C, followed by centrifugation at room temperature for 30 min at 16100 g. Supernatants were discarded and pellets were resuspended in 1× NuPAGE LDS denaturing sample buffer (Invitrogen) with 3% SDS added and heated to 100°C for 8 min before gel electrophoresis. Samples were loaded on preformed 1 mm 4–12% Bis/Tris SDS-PAGE denaturing, reducing, polyacrylamide gradient gels in MOPS/SDS running buffer (NuPAGE gel system; Invitrogen) and electrophoresed for 1 h at 200 V. Molecular mass markers were MagicMark XP Western protein standard (Invitrogen).

Samples from gels were transferred electrophoretically to Immobilon-P PVDF membrane (Millipore) in 25 mM Tris, 192 mM glycine, 10% methanol by using a Hoefer TE22 Mighty Small Transphor tank (Amersham Biosciences) at 400 mA for 1 h with stirring and cooling of transfer buffer. Following transfer, membranes (blots) were rinsed briefly in 100% methanol and air-dried. Preceding Western immunoblot development, dried blots were rinsed for 15 s in 100% methanol and for 2 min in distilled water and placed in 30 ml blocking buffer [50 mM Tris/HCl (pH 7·5), 150 mM NaCl, 0·1% Tween 20 (Bio-Rad) (TBS-T)], containing 4% non-fat dry milk (NFDM) (Carnation) and 2 mg normal goat serum ml\textsuperscript{-1} (Jackson ImmunoResearch Laboratories) for 30 min at room temperature. Blots were subsequently incubated 1 h with anti-PrP antibody (mouse monoclonal F99/97·6·1; VMRD Inc.) diluted 1:200 in TBS-T/NFDM, followed by three 10 min washes in 50 ml TBS-T per wash and a second incubation in blocking buffer for 5 min, before incubating for 30 min with horseradish peroxidase-conjugated F(ab')\textsubscript{2} fragment of goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) diluted 1:30000 in TBS-T/NFDM. Secondary antibody incubation was followed by five 10 min washes in TBS-T, chemiluminescent detection by using ECL Western blotting reagents (Amersham Biosciences) according to the manufacturer’s directions and exposure of Kodak BioMax ML film for times ranging from 1 min to overnight, followed by manual film development. Digitized images for figures were obtained by scanning developed films and photoediting with HP Director software; photoediting was limited to cropping and labelling images and adjustments in brightness and contrast.

**IHC.** Striated muscle tissues collected at necropsy were preserved in 10% neutral-buffered formalin. Samples were trimmed into sections, treated in formic acid, rinsed, dehydrated, paraform-embedded, prepared for immunohistochemical staining and stained, all as described previously (Miller & Williams, 2002), with the following modifications: immersion in 98% formic acid of sections on slides to enhance epitope exposure was for 5 min, followed by a rinse in 50 mM Tris/HCl (pH 7·6), 0·15 M NaCl, then deionized water; cooling after autoclaving was for 40 min. Primary antibody was...
mAb F99/97.6.1 [Anti-Prion (99); Ventana Medical Systems]. Digitized images for figures were obtained by using an Olympus BX51 light microscope equipped with a MicroFire digital camera and PictureFrame for Windows software to view and photograph slides. Photomontage (Microsoft Office Picture Manager) was limited to cropping and labelling images and adjustments in brightness, contrast or colour balance.

Animals and tissues. Samples were of muscle tissues that had been collected at necropsy at the WSVL and frozen. Animals included free-ranging deer collected for CWD surveillance and submitted by WGFD to the WSVL for necropsy and testing, and captive or free-ranging animals that were subjects in other CWD research projects carried out in collaboration with WGFD and/or the Colorado Division of Wildlife. All elk and moose were experimental animals from WGFD research.

RESULTS

ELISAs

To investigate whether PrPd was present in striated muscle of CWD-infected cervids, we first assayed muscle samples from 15 mule deer (ten CWD-infected and five uninfected), nine white-tailed deer (four infected, five uninfected) and 13 elk (ten infected, three uninfected) by ELISA (see Methods). We used six muscle samples from each animal: diaphragm, tongue, left ventricle (lv) of the heart and three appendicular skeletal muscles (longissimus dorsi, triceps brachii and semitendinosus). lv from two CWD-positive white-tailed deer and seven CWD-positive elk resulted in positive ELISA, but all other samples were negative (Fig. 1).

Western blots

We confirmed these results by Western blots using new homogenates from the same tissues as were used for ELISA for the 24 CWD-positive animals and seven of the negative controls. The same six muscles were also sampled from one additional CWD-positive white-tailed deer and from two moose. In addition, lv only from seven CWD-infected elk, three CWD-infected mule deer and eight CWD-infected white-tailed deer was assayed by Western blot. The presence of PK-resistant fragments of PrPd that exhibited the characteristic electrophoretic pattern of three bands corresponding to the three glycosylated states of PrP and that migrated between approximately 35 and 20 kDa was demonstrated by Western blot in the cardiac lv of specimens that were lv-positive by ELISA (Figs 2 and 4). For samples tested only in lv, PK-resistant PrP was found in five of seven elk, two of eight white-tailed deer and none of three mule deer. Results are summarized in Table 1. The relative concentration of PrPd in lv was estimated visually from band intensity and comparison of tissue weights per sample lane to be roughly 10- to 100-fold lower than that demonstrated in brain tissue of the same animals (Fig. 3). Because both brain and heart samples can exhibit locational sampling variation as shown, more detailed estimates of concentrations were

<table>
<thead>
<tr>
<th>Mule deer</th>
<th>White-tailed deer</th>
<th>Elk</th>
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Fig. 1. Summary of ELISA results for detection of PrPd in deer and elk striated muscles. Muscle tissues: tongue (TG), triceps (TR), left ventricle (LV), diaphragm (DI), longissimus dorsi (LD) and semitendinosus (ST). Assays were performed as described in Methods. Horizontal dashed lines mark cut-off values for positive ELISA. Symbols of the same geometric shape with interior patterns are retests of the same specimen.
not made. We did not detect PrP\textsuperscript{d} by Western blot in any of the other muscles tested in any of the animals, including uninfected controls (data not shown).

We prepared homogenates from other sites in the lv tissue of the CWD-positive animals and assayed them by Western blot to test the possibility of an inhomogeneous distribution of PrP\textsuperscript{d} (Glatzel et al., 2003). If PrP\textsuperscript{d} were associated primarily with cells of the cardiac conduction system or with intramural cardiac ganglia, we reasoned that such arrangements could produce 'hit-or-miss' sampling and might account for results that showed both lv-positive and lv-negative specimens among the CWD-positive animals. We observed variable intensity of the PK-resistant PrP\textsuperscript{d} bands from lv-positive animals (Fig. 4), particularly some of the elk samples, indicating uneven but dispersed distributions of PrP\textsuperscript{d} within the ventricular tissue. However, we were unable to demonstrate the presence of PrP\textsuperscript{d} in the lv tissue of any mule deer, moose or three white-tailed deer and three elk that had previously been negative.

IHC

Inspection of sections of fixed lv tissue by IHC revealed anti-PrP\textsuperscript{d}-labelled groups of laterally adjacent muscle cells within the ordinary myocardium, often surrounded by cells with no detectable stain (Fig. 5a, b). Labelled antigen was more readily visible in longitudinal than in transverse cross-sections of cardiac myocytes. Staining generally consisted of punctate granules of chromagen scattered in an apparently cell-associated fashion (Fig. 5c), sometimes in linear patterns along the edge of a muscle cell (Fig. 5d), but not consistently so. Subcellular structures or nerve fibres with which PrP\textsuperscript{d} might have been associated were not identified. Epicardial nerves and ganglia and subendocardial and myocardial Purkinje fibres lacked detectable PrP\textsuperscript{d} staining. Staining of PrP\textsuperscript{d} in elk myocardium was similar to that of white-tailed deer (Fig. 5e).

We did not detect PrP\textsuperscript{d} by IHC in striated muscles other than heart. Staining was equivocal or lacking in the skeletal muscles and diaphragm sections. Light staining in small ganglia of the tongue was observed in a few specimens of white-tailed deer and elk, and two small groups of ventricular subendocardial Purkinje fibres in the heart.

### Table 1. Summary of heart tissue samples tested

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<thead>
<tr>
<th>Species</th>
<th>lv-positive*</th>
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<td>Mule deer</td>
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<tr>
<td>CWD-positive</td>
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</tr>
<tr>
<td>CWD-negative</td>
<td>0/5</td>
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<tr>
<td>Elk</td>
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</tr>
<tr>
<td>CWD-positive</td>
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</tr>
<tr>
<td>CWD-negative</td>
<td>0/3</td>
</tr>
<tr>
<td>White-tailed deer</td>
<td></td>
</tr>
<tr>
<td>CWD-positive</td>
<td>7/16</td>
</tr>
<tr>
<td>CWD-negative</td>
<td>0/6</td>
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*lv, Left ventricle; no. positive in lv/no. tested by ELISA, Western blot or IHC.

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**Fig. 2.** Immunoblot assays for detection of PK-resistant PrP in heart lv of CWD-infected white-tailed deer (WTD) and elk. Comparison of PrP\textsuperscript{d} from 10 mg wet tissue equivalents of lv of WTD and elk. Film exposure to enhanced chemiluminescence was 80 min.

**Fig. 3.** Comparison of PrP\textsuperscript{d} from brain and lv of elk (lanes 1–8) and white-tailed deer (WTD) (lanes 9–16). Lanes: 1, elk brain, 1 mg tissue equivalent; 2, elk lv, 10 mg tissue; 3, elk brain, 0.1 mg (lanes 1, 2 and 3 are from the same blot; samples between lanes 1 and 2 were cut from image); 4, elk lv –PK; 5, elk lv +PK; 6, elk lv +PK; 7, elk brain +PK; 8, elk brain –PK; 9, WTD brain +PK; 10, WTD brain sample 1, 1 mg; 11, WTD brain sample 2, 1 mg; 12, WTD brain sample 3, 1 mg; 13, WTD lv, 5 mg; 14, WTD lv, 1 mg; 15, WTD lv –PK; 16, WTD lv +PK. M, Molecular mass markers (bottom to top, approx. 20, 30 and 40 kDa).

**Fig. 4.** Heterogeneous PrP\textsuperscript{d} distribution in lv. Lanes a, b, c and d were sampled from different sites in the lv tissue of the same animal. WTD gel samples were 20 mg wet tissue equivalents; film exposure was 10 min. Elk gel samples were 10 mg tissue equivalents; exposure time was 145 min.
of the CWD-positive moose were stained for PrP^d (data not shown).

**Distribution of PrP^d in heart**

By using complete hearts collected and frozen at necropsy from two captive CWD-affected white-tailed deer (the generous gift of Dr L. L. Wolfe, Colorado Division of Wildlife, Denver, CO, USA) and from a CWD-affected free-ranging white-tailed deer (the generous gift of D. Edmunds, University of Wyoming, Laramie, WY, USA), we tested other areas of the heart by Western blot to investigate how restricted or widespread PrP^d accumulation might be. One specimen (WTD21) was positive for PrP^d throughout the right atrium and strongly so in the free wall of both ventricles (Fig. 6a); another (WTD22) was weakly positive only in atrial tissue taken from the terminal crest between the right auricle and atrium, and in the interatrial septum adjacent and cranial to the opening of the coronary sinus in the area of the atrioventricular node (Fig. 6b). Heart of the free-ranging white-tailed deer (WTD20) was positive in all atrial areas sampled, strongly so in the region of the sinoatrial node, but negative in the free wall of the lv (Fig. 6c). These results indicated that PrP^d could be present in atrial tissue without being detected in ventricular muscle. Our results with lv tissues probably underestimated the frequency of PrP^d deposition in cardiac tissues of CWD-affected white-tailed deer. By using the complete heart from a mule deer that died in a late stage of CWD (MD18), we assayed 16 sites in atria and ventricles by Western blot for PrP^d. All samples were negative (Fig. 6d, e). No complete hearts were available from CWD-affected elk.

**DISCUSSION**

We have demonstrated by three immunological assays that PrP^d is found in cardiac tissue of CWD-infected cervids. We failed to detect PrP^d by the same assays in the five other striated muscles tested, which suggested that PrP^d did not accumulate there, but which by the nature of negative evidence is inconclusive. We cannot rule out the possibility that some PrP^d is present in other striated muscles not examined or might be detectable by other assays, including bioassay by inoculation of conspecific animals or of transgenic mice. Recently, Angers et al. (2006) reported the observation of TSE in transgenic mice following i.c. inoculation with extracts of semitendinosus muscle from CWD-infected mule deer. Similar tests of skeletal muscles from CWD-infected white-tailed deer or elk have not yet been reported.

The negative evidence in the case of mule deer heart is somewhat more convincing, because positive heart samples from white-tailed deer and elk are essentially positive controls by which the lack of PrP^d in mule deer heart can be validated for each assay. In addition, our negative results for mule deer heart are in agreement with an earlier investigation using IHC (Spraker et al., 2002a). Results of experiments to demonstrate the infectivity of cardiac-associated PrP^d by bioassays, as well as to test for infectivity in mule deer heart, are not yet completed.

It was not known previously that PrP^d accumulated in cardiac muscle of any TSE-infected animals and several questions are raised by these findings. One question is whether impairment of cardiac function is associated with prion deposition in myocardium. For the human spongiform encephalopathies, one case has been reported of PrP^d detected by IHC in an endomyocardial biopsy sample from a Creutzfeld–Jakob disease patient with dilated cardiomyopathy (DCM) (Ashwath et al., 2005). The authors of that report suggested the possibility of prion-induced DCM on the basis of finding no other causes for the condition after an exhaustive search. Neither DCM nor other gross lesions in heart of CWD-infected deer and elk have been commonly observed at necropsy (E. S. Williams, unpublished observations; T. E. Cornish, personal communication). Disturbances in heart rate have been identified in BSE-infected cattle (Austin et al., 1997; Pomfrett et al., 2004), but were attributed possibly to associated neuropathology in the parasympathetic centres of cardiac innervation; neither
infectivity nor PrPSc has been detected in heart of BSE-infected cattle (Wells et al., 1996; European Commission Scientific Steering Committee, 2002; Buschmann & Groschup, 2005; Wells et al., 2005). Similar data from cardiac monitoring of CWD-infected cervids are not available, but could be informative.

It remains to be determined whether the prions that we detected in cardiac tissue were localized to nerve fibres or subcellular structures within myocytes. For this study, our IHC examination of cardiac tissues from CWD-infected animals was restricted to lv. The grouped arrangement of PrP-labelled myocytes that we found in ventricular muscle might be the result of prion movement along nerve fibres that correspond to a particular subpopulation of neurons innervating the heart. Further investigation, such as co-labelling studies, will be required for a better understanding of the structural arrangements underlying our observations.

It will also be necessary to examine by IHC sites throughout the heart such as were analysed in frozen tissues by Western blot, in order to find out what the distribution of PrPd is and, possibly, where it appears first. Many of the heart-positive animals were in clinical stages of disease, but the time during infection at which PrPd appears in heart tissue has not been determined. Although discussions of routes by which PrPd reaches the heart are speculative in nature at this time, if it appears only after having progressed to the brain and spinal cord, this would suggest secondary dissemination from CNS to the heart, possibly via efferent parasympathetic and sympathetic nerves that innervate the heart from parasympathetic nuclei of the ventrolateral medulla of the brainstem (nucleus ambiguus primarily) (Loewy & Spyer, 1990; Standish et al., 1994) and from sympathetic preganglionic neurons that arise in the intermediolateral cell column of rostral sections of the thoracic spinal cord (McKibben & Getty, 1969a, b, 1970; Cabot, 1990). Both autonomic cardiac-control centres are among neural sites that accumulate PrPd during CWD infection following initial deposition and propagation in the dorsal motor nucleus of the vagus and in the spinal cord (Sigurdson et al., 1999, 2001; Peters et al., 2000; Spraker et al., 2002b).

Finally, determining what differential factors in these closely related species are responsible for the appearance of PrPd in heart of elk and white-tailed deer, but not of mule deer, may provide important insights into variables that promote or inhibit prion trafficking along neural conduits during a TSE infection.

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