

# PrP<sup>CWD</sup> in the myenteric plexus, vagosympathetic trunk and endocrine glands of deer with chronic wasting disease

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Accumulated evidence in experimental and natural prion disease systems supports a neural route of infectious prion spread from peripheral sites of entry to the central nervous system. However, little is known about prion trafficking routes in cervids with a naturally occurring prion disease known as chronic wasting disease (CWD). In the brain, the pathogenic isoform of the prion protein (PrP<sup>CWD</sup>) accumulates initially in the dorsal motor nucleus of the vagus nerve. To assess whether alimentary-associated neural pathways may play a role in prion trafficking, neural and endocrine tissues from mule deer naturally infected with CWD ( $n = 6$ ) were examined by immunohistochemistry. PrP<sup>CWD</sup> was detected in the myenteric plexus, vagosympathetic trunk, nodose ganglion, pituitary, adrenal medulla and pancreatic islets. No to scant PrP<sup>CWD</sup> staining was detected in other nerves or ganglia (brachial plexus, sciatic nerve, gasserian ganglion, coeliac ganglion, cranial cervical ganglion, spinal nerve roots) of CWD-positive deer and no PrP<sup>CWD</sup> was detected in nerves or endocrine tissues from 11 control deer. These findings suggest that: (i) transit of PrP<sup>CWD</sup> in nerves, either centrifugally or centripetally, is one route of prion trafficking and organ invasion and (ii) endocrine organs may also be targets for cervid pathogenic prion accumulation.

## Introduction

Chronic wasting disease (CWD) is an endemic transmissible spongiform encephalopathy (TSE) of captive and free-ranging mule deer, white-tailed deer and Rocky Mountain elk in Colorado and Wyoming (Williams & Young, 1980, 1982; Spraker *et al.*, 1997). CWD is transmitted efficiently in nature, with the prevalence reaching 15% in some subpopulations (Miller *et al.*, 2000). Relatively little is known, however, about the mode of transmission or the pathogenesis of this naturally occurring TSE. TSEs are characterized by the accumulation of a pathogenic, partially protease-resistant isoform (PrP<sup>res</sup>) of a normal cellular protein (PrP<sup>c</sup>) (Prusiner, 1982). PrP<sup>res</sup> deposition occurs principally in the central nervous system (CNS), resulting in a neurodegenerative disease and in some prion infections, e.g. scrapie and variant

Creutzfeldt–Jakob disease (vCJD), PrP<sup>res</sup> deposition or infectivity also occurs in lymphoid tissues (Hadlow *et al.*, 1982; Hill *et al.*, 1999; Brown *et al.*, 1999). Understanding routes of agent spread from peripheral entry sites to the CNS is fundamental to developing strategies to block neuroinvasion.

A growing body of evidence including both experimental and natural studies strongly implicates PrP<sup>res</sup> dissemination from the entry site via peripheral nerves. Pioneering studies by Kimberlin and colleagues in intrasciatically or intragastrically inoculated mice demonstrated scrapie transport from the peripheral nervous system (PNS) directly to the brain or from the intestinal tract to the thoracic spinal cord via splanchnic nerves (Kimberlin *et al.*, 1983; Kimberlin & Walker, 1989). Additionally, in hamsters challenged orally with 263K scrapie, PrP<sup>res</sup> was detected by immunohistochemistry (IHC) in early infection within the myenteric and submucosal plexuses (Beekes & McBride, 2000) as well as the dorsal motor nucleus of the vagus nerve (DMNV) in the brain (Beekes *et al.*, 1998) and terminally in the vagus nerve and the nodose, dorsal root

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and coeliac ganglia, findings consistent with spread via vagal and splanchnic routes (McBride & Beekes, 1999). van Keulen *et al.* (1999, 2000) demonstrated PrP<sup>res</sup> deposits in the enteric nervous system in natural sheep scrapie, which supported the alimentary tract as the site of neural invasion. Moreover, nerve infectivity or PrP<sup>res</sup> has been detected in natural (Hadlow *et al.*, 1982; Groschup *et al.*, 1996) and experimental (Groschup *et al.*, 1999) scrapie, BSE-infected lemurs (Bons *et al.*, 1999) and in a natural case of CJD (Hainfellner & Budka, 1999).

Recent findings have highlighted the significance of spread via peripheral nerves. Glatzel & Aguzzi (2000) compared PrP<sup>res</sup> tissue distribution patterns in wild-type versus transgenic PrP<sup>c</sup>-overexpressing mice. They demonstrated that elevated PrP<sup>c</sup> expression in the PNS biased PrP<sup>res</sup> transit pathways toward intranervous spread in transgenic mice versus lymphoreticular spread in wild-type mice. Race *et al.* (2000) demonstrated that transgenic mice expressing hamster PrP<sup>c</sup> in neural but not lymphoid tissues developed brain PrP<sup>res</sup> infection after oral or intraperitoneal inoculation with hamster scrapie, establishing a vital role for PrP<sup>c</sup> peripheral nerve expression in neuroinvasion.

As with other prion diseases (scrapie, BSE, kuru and vCJD), CWD infections are suspected to arise from oral exposure to the causative agent. In deer infected orally with brain containing PrP<sup>res</sup> (PrP<sup>CWD</sup>), PrP<sup>CWD</sup> deposition was first detected in alimentary-associated lymphoid tissues (Sigurdson *et al.*, 1999) and subsequently in the DMNV in the medulla oblongata (Williams & Miller, 2000). In naturally infected deer, histological lesions of TSE and PrP<sup>CWD</sup> were present in the hypothalamus, thalamus, brainstem and spinal cord grey matter with smaller amounts in the cerebral cortex and cerebellum (Spraker *et al.*, 1997, 2001). PrP<sup>res</sup> correlates closely with infectivity and serves as a surrogate marker for prion infection (McKinley *et al.*, 1983; Race *et al.*, 1998). Thus, IHC can be used to localize PrP<sup>res</sup> in tissues.

Because the DMNV is the initial target site for PrP<sup>CWD</sup> in the brain (Williams & Miller, 2000) and the vagosympathetic trunk carries vagal nerve fibres that innervate the alimentary tract, we examined alimentary nerves and ganglia from mule deer with naturally occurring CWD by using IHC. For comparison with non-alimentary nerves, we examined the brachial plexus and the sciatic nerve, which respectively innervate the forelimb and hindlimb. In order to investigate components of the sympathetic splanchnic circuitry, coeliac ganglia and thoracic spinal cord with associated nerve roots were assessed. Gasserian ganglia were included to explore potential PrP transit via the trigeminal nerve. We report that the myenteric plexus, vagosympathetic trunk and, to a lesser degree, the other peripheral nerves of deer contain PrP<sup>CWD</sup>, indicating that nerve transport may be one route of PrP trafficking in CWD. An unexpected finding was the detection of PrP<sup>CWD</sup> in pancreatic islet cells, adrenal medulla and the pituitary, suggesting nerve-vectored transit may also occur to endocrine organs.

## Methods

**■ CWD-infected deer and tissue collection.** Six captive mule deer (*Odocoileus hemionus*) with naturally occurring, clinical CWD were euthanized and the following tissues were collected for assessment by IHC: brain, ~15 cm of the cervical vagosympathetic trunk, 15–20 cm of the sympathetic trunk from the thoracic vertebral region, 8–10 cm of the sciatic nerve, 8–10 cm of the brachial plexus, a 4 cm<sup>2</sup> section of pancreas, the pituitary, adrenal gland, small intestine, coeliac and gasserian ganglia and thoracic spinal cord (Fig. 1). Tissues were fixed in 10% neutral-buffered formalin for 1–3 days and then immersed in 88% formic acid for 1 h and embedded in paraffin.

**■ Negative-control deer and tissues.** Brain and vagosympathetic trunk from eight free-ranging mule deer from a CWD non-endemic area (non-endemic area established by methods of Miller *et al.*, 2000) and brain, sciatic nerve, adrenal gland, pancreas, small intestine, coeliac and gasserian ganglia, thoracic spinal cord and pituitary of three mule deer inoculated with CWD-negative brain homogenate from a previous study (Sigurdson *et al.*, 1999) were similarly fixed and processed.

**■ IHC staining.** Tissue sections were mounted onto positively charged glass slides, deparaffinized, hydrated, autoclaved in a citrate buffer solution (DAKO Target Antigen Retrieval) for 20 min at 121 °C and cooled for 5 min.

The IHC protocol employed an automated immunostainer (Ventana Medical Systems) and anti-PrP MAb 6H4 or 99/97.6.1 (generously provided by K. O'Rourke), a biotinylated secondary antibody, an alkaline phosphatase–streptavidin conjugate, a substrate chromogen (fast red A) and a haematoxylin and bluing counterstain (Ventana Medical Systems). MAb 6H4 recognizes a conserved sequence of the prion protein, corresponding to residues 144–152 of the human amino acid sequence,

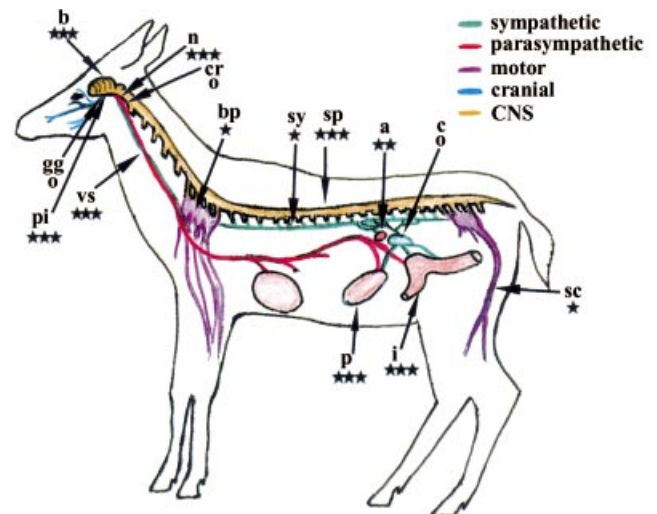
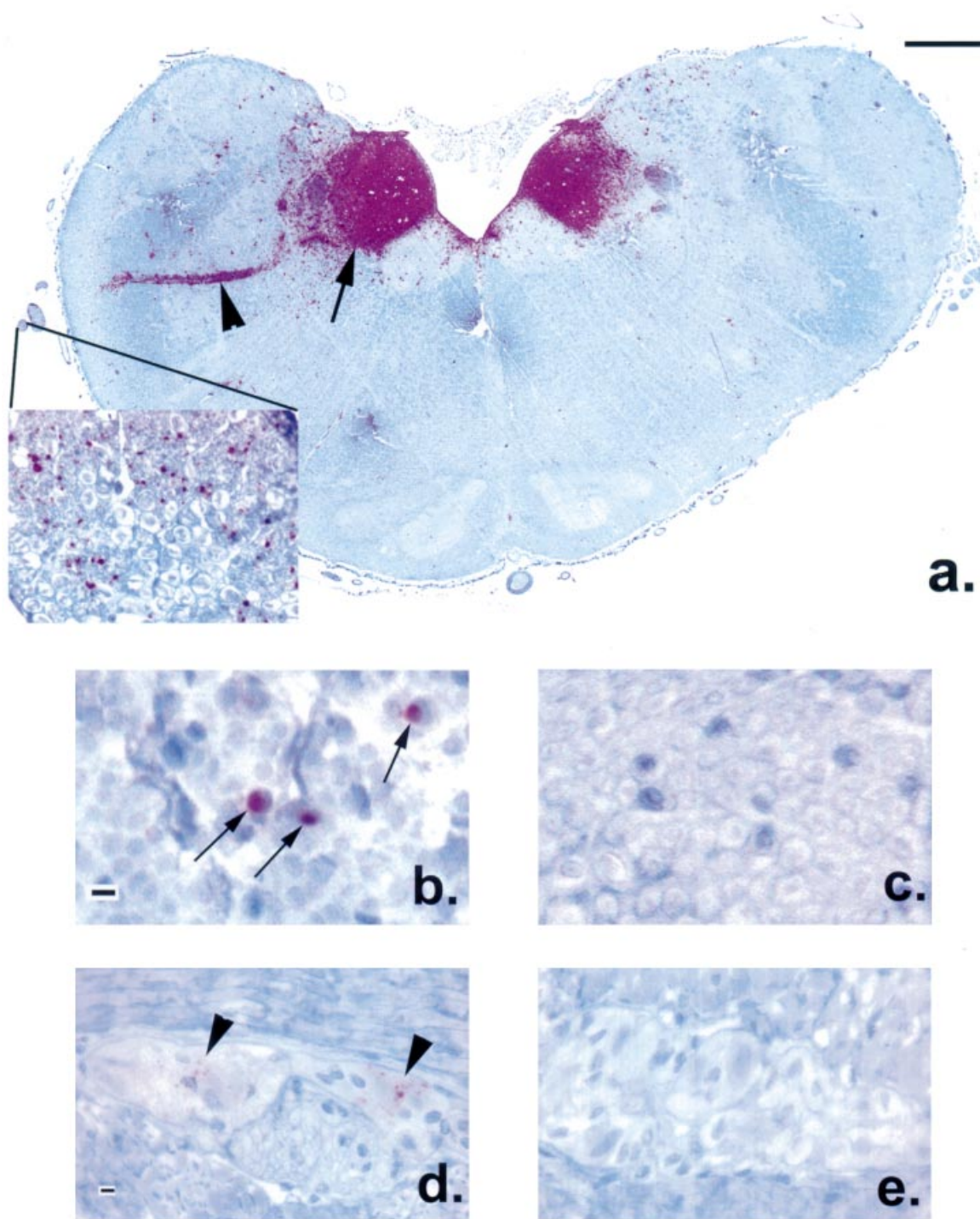


Fig. 1. Distribution of sympathetic, parasympathetic, cranial and motor nerves examined for the pathogenic isoform of the prion protein. Arrows indicate sites sampled. Number of stars indicates the incidence of PrP<sup>CWD</sup> IHC positivity in the six deer, defined as the percentage positive of the total for each tissue sampled (0, 0%; \*, 1–50%; \*\*, 51–75%; \*\*\*, 75–100%). Abbreviations: b, brain; n, nodose ganglion; cr, cranial cervical ganglion; bp, brachial plexus; sy, sympathetic trunk; sp, spinal cord; a, adrenal; c, coeliac ganglion; sc, sciatic; p, pancreas; i, intestine/myenteric plexus.



**Fig. 2.** IHC detection of PrP<sup>CWD</sup> in the central and peripheral nervous system of naturally infected mule deer using MAb 6H4. (a) Brain at medulla oblongata. PrP<sup>CWD</sup> stain in the DMNV (arrow), vagal radix (arrowhead) and presumably the vagus nerve exiting the section (inset). (b)–(e) PrP<sup>CWD</sup> labelling was detected in the vagosympathetic trunk (b, arrows) and in the myenteric plexus of the small intestine (d, arrowheads) of CWD-infected deer, but not in the control CWD-negative deer (c, e). Bars, 1 mm (a) or 10 μm (b, d).

and recognizes PrP epitopes of rabbit, mink, sheep, cattle and primates (Korth *et al.*, 1997). MAb 99/97.6.1 recognizes residues 220–225 of the ovine prion protein (O'Rourke *et al.*, 2000). An isotype-matched,

irrelevant antibody was substituted in the IHC protocol as a negative control. Each immunostained nerve section was examined once or twice with each antibody.

## Results

### PrP<sup>CWD</sup> in nerve and ganglia

Given that alimentary exposure is likely in CWD, we focused on two major autonomic nerve tracts as potential PrP<sup>CWD</sup> conduits: (i) the vagosympathetic trunk and (ii) the splanchnic neural circuitry. The vagosympathetic trunk includes parasympathetic vagal nerve fibres, which have nerve cell bodies in the DMNV, pass through the nodose ganglion and synapse with the myenteric plexus of the small intestine.

The splanchnic nerves, which have nerve cell bodies in the intermediolateral cell column of the thoracic spinal cord, carry sympathetic fibres that synapse directly in the adrenal medulla or synapse in the coeliac ganglion and innervate the oesophagus, stomach and small intestine (Fig. 1).

CWD-positive deer were diagnosed by: (i) histological lesions of CWD in the medulla oblongata including perikaryonic neuronal vacuoles, spongiform degeneration of the neuropil and astrocytosis, and (ii) abundant PrP<sup>CWD</sup> stain in the DMNV by IHC. Deer were confirmed as CWD-negative by

**Table 1.** IHC detection of PrP<sup>CWD</sup> in nerves from CWD-infected mule deer

MAbs 6H4 and 99/97.6.1 were used to detect the prion protein. IHC stain was quantified as the number of positive stain granules in a section: +, < 10; ++, 10–20; +++, > 20. ND, Not done.

Tissue	MAb	Deer case number						Positive (%)
		V92	Za93	B93	Mb97	W97	H92	
Vagosympathetic trunk	6H4	+	+	+	++	++	++	100
Vagosympathetic trunk	99/97.6.1	+	++	–	++	+++	+	83
Sciatic nerve	6H4	–	ND	–	+	–	–	20
Sciatic nerve	99/97.6.1	–	ND	–	++	–	–	20
Sympathetic trunk	6H4	–	ND	–	–	+	ND	25
Sympathetic trunk	99/97.6.1	–	ND	–	–	++	ND	25
Brachial plexus	6H4	–	–	ND	ND	–	–	0
Brachial plexus	99/97.6.1	–	++	ND	ND	–	–	25

**Table 2.** IHC detection of PrP<sup>CWD</sup> in neural and endocrine tissues from CWD-infected mule deer

MAbs 6H4 and 99/97.6.1 were used to detect the prion protein. ND, Not done.

Tissue	MAb	Deer case number						Positive (%)
		V92	Za93	B93	Mb97	W97	H92	
Medulla oblongata	6H4	+	+	+	+	+	+	100
Medulla oblongata	99/97.6.1	+	+	+	+	+	+	100
Intermediolateral cell column of spinal cord	99/97.6.1	+	ND	ND	+	+	+	100
Pituitary	6H4	+	+	+	+	+	+	100
Pituitary	99/97.6.1	+	+	+	+	+	+	100
Pancreas	6H4	+	+	–	+	+	–	67
Pancreas	99/97.6.1	+	+	+	+	+	–	83
Adrenal medulla	99/97.6.1	ND	–	+	–	+	+	60
Myenteric plexus	99/97.6.1	+	+	–	+	+	+	83
Coeliac ganglion	99/97.6.1	ND	ND*	–	–	–	–	0
Nodose ganglion	99/97.6.1	ND	+	ND	ND	+	ND	100
Cranial cervical ganglion	99/97.6.1	ND	ND	ND	–	ND	ND	0
Gasserian ganglion	99/97.6.1	–	ND*	–	–	–	–	0
Spinal nerve roots, dorsal and ventral	99/97.6.1	–	ND	ND	–	–	–	0

\* Associated nerve was negative.



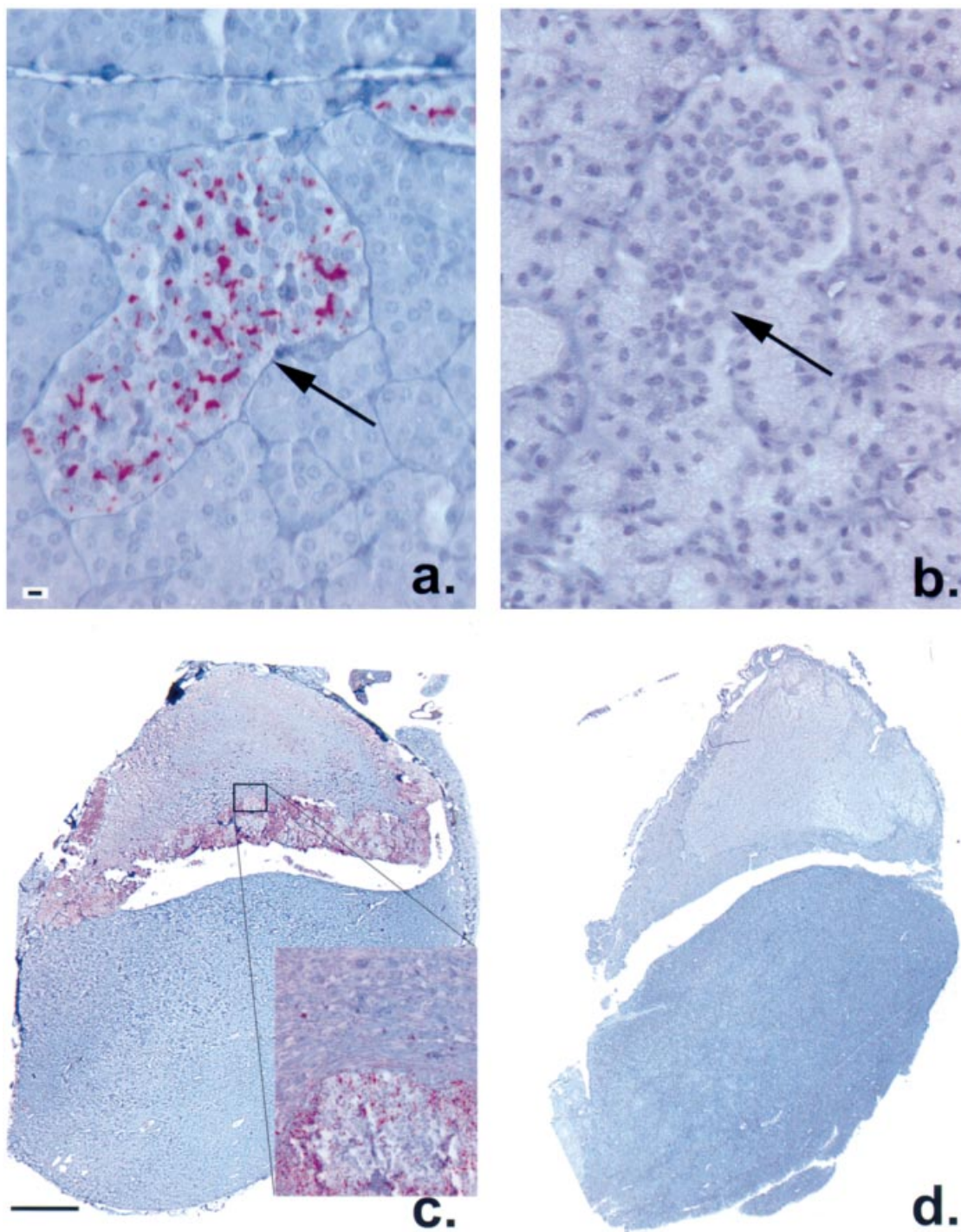


Fig. 3. IHC detection of PrP<sup>CWD</sup> in endocrine organs of naturally infected mule deer. Pancreatic islets of Langerhans (arrows) accumulate PrP<sup>CWD</sup> in CWD-infected (a) but not uninfected (b) deer. Pars intermedia and nervosa of the pituitary accumulate PrP<sup>CWD</sup> in CWD-infected (c) but not control (d) deer. Bars, 10  $\mu$ m (a) or 1 mm (c).

the absence of histological lesions and negative staining for PrP<sup>CWD</sup> in all tissues. In one deer, abundant PrP<sup>CWD</sup> stain was present in the DMNV, the radix tract and the vagus nerve exiting the obex (Fig. 2a). PrP<sup>CWD</sup> was detected in the vagosympathetic trunk ( $n = 6$  deer), sciatic nerve ( $n = 1$ ,

sympathetic trunk ( $n = 1$ ) and brachial plexus ( $n = 1$ ) (Table 1). The stain deposits appeared as scattered coarse granules within the nerve fascicles and occasionally within axons (Fig. 2b). The staining detected in the sciatic nerve and brachial plexus was scant compared with the vagosympathetic trunk.

No stain deposits were visible in the nerves from CWD-negative deer from known non-endemic geographical regions using MAb 6H4 (Fig. 2*c*). Three stain granules were detected in one nerve of one negative-control deer using MAb 99/97.6.1; therefore only nerves with more than three stain granules were considered positive. PrP<sup>CWD</sup> was present in the myenteric plexus ( $n = 5$ , Fig. 2*d*), nodose ganglion ( $n = 2$ ) and in the intermediolateral cell column of the thoracic spinal cord ( $n = 4$ ), but not in the cranial cervical, coeliac or gasserian ganglia (Table 2). Stain deposits in the nodose ganglia appeared primarily along nerve fibres and in satellite cells with little to no stain within the ganglion cell body. However, coarse stain deposits were present in the myenteric plexus neurons (Fig. 2*d*).

Minor differences in tissue-staining positivity were observed between MAbs 6H4 and 99/97.6.1 (Tables 1 and 2). The IHC stain deposits in nerve were irregularly distributed and widely spaced along the fascicle. Stain was quantified in the nerve only by the number of red PrP stain granules present in a section and recorded as + (four to ten), ++ (ten to twenty) or +++ (more than twenty). Nerve tissues incubated with an irrelevant MAb were negative.

#### PrP<sup>CWD</sup> deposits in pituitary, islets of Langerhans and adrenal medulla

The pancreases of five of six CWD-positive deer contained diffuse, coarse granular PrP<sup>CWD</sup> deposits confined to the islets of Langerhans (Fig. 3*a*). Although fewer than half of the islets were affected in any pancreas section in four of five deer, PrP<sup>CWD</sup> stain was abundant in the affected islets. Such deposits were not detected in pancreases of CWD-negative deer (Fig. 3*b*). In the pituitaries of all CWD-positive deer, PrP<sup>CWD</sup> deposits were evident, primarily in the pars nervosa and intermedia (Fig. 3*c*), and were not seen in the CWD-negative deer (Fig. 3*d*). Likewise, PrP<sup>CWD</sup> staining was identified in the adrenal medulla in three of five CWD-positive deer and not the controls.

#### Discussion

We detected PrP<sup>CWD</sup> in the myenteric plexus, vagosympathetic trunk and intermediolateral cell column of the spinal cord of naturally infected CWD deer, consistent with previous findings in experimental and natural TSE. Likewise, scrapie PrP<sup>res</sup> has been demonstrated in submucosal and myenteric plexuses in orally inoculated hamsters (Beekes & McBride, 2000) and in naturally infected sheep (van Keulen *et al.*, 2000). These findings suggest that prion trafficking may occur by centripetal or centrifugal nerve transport. In orally challenged deer euthanized sequentially from 3–28 months post-inoculation ( $n = 20$ ), PrP<sup>CWD</sup> was detected initially within the DMNV of the brain by IHC (Williams & Miller, 2000). The initial appearance of PrP<sup>CWD</sup> in the DMNV implicates the vagus nerve as a potential route for PrP<sup>CWD</sup>

transit from the presumed site of exposure in the alimentary tract to the CNS.

Abundant PrP<sup>CWD</sup> was detected in the vagosympathetic trunk and in nerve fibres in the nodose ganglion, compared with scant or no deposition of PrP in the cranial cervical ganglion (sensory), coeliac ganglion, sciatic nerve or brachial plexus, which suggests that the vagus nerve could serve as a major transit route of PrP<sup>CWD</sup>. PrP<sup>CWD</sup> was detected in myenteric ganglion cell bodies, along nerve fibres and in satellite cells, as has been described in other studies (Groschup *et al.*, 1999; McBride & Beekes, 1999). Nevertheless, other routes of PrP<sup>CWD</sup> transit, such as via blood, sensory or cranial nerves innervating the oral mucosa (IX, X) or sympathetic splanchnic nerves, cannot be excluded. In haematogenous dissemination, it might be expected that PrP<sup>CWD</sup> amplification would occur initially in richly vascular neural domains with fenestrated endothelium (e.g. area postrema of the medulla oblongata, hypophysis, pineal body, hypothalamic regions, subfornical organ) as opposed to the DMNV. Dissemination via cranial nerves IX and X might be expected to result in initial PrP<sup>CWD</sup> amplification in the nucleus solitarius.

The gastrointestinal tract receives parasympathetic vagal nerve fibres from the DMNV and sympathetic nerve fibres from the spinal cord via the coeliac ganglion (splanchnic circuitry). In the light of PrP<sup>CWD</sup> detection in the intermediolateral column of the spinal cord and adrenal medulla, which is innervated by splanchnic nerves, it is plausible that PrP<sup>CWD</sup> may also traffic to the CNS via the splanchnic circuitry. If this is the case, it is surprising that we did not detect PrP<sup>CWD</sup> in the coeliac ganglion, in which pre-ganglionic splanchnic nerve fibres to the intestine synapse. It is possible that PrP<sup>CWD</sup> in the intermediolateral column resulted from spread within the CNS and then spread centrifugally to the adrenal medulla, a route that does not involve the coeliac ganglion.

Neurotropic viruses that enter the host via the gastrointestinal tract have been investigated to determine their route of entry into the CNS. The pathogenesis of prion infections has been compared to that of pseudorabies virus (Beekes *et al.*, 1998), which spreads retrograde along parasympathetic vagal efferents to the DMNV (Card *et al.*, 1990). Similarly, in mice inoculated perorally with a neurotropic reovirus, the virus spread to the myenteric plexus and then retrograde along the vagus efferent nerve to the DMNV, regardless of the amount of virus in the bloodstream. Moreover, subcutaneous inoculation over the forehead resulted in the detection of virus in the facial and trigeminal nuclei of the brain, but not in the DMNV, establishing that detection of virus in the DMNV depended on an oral inoculation route (Morrison *et al.*, 1991).

Substantial deposits of PrP<sup>CWD</sup> were detected in the pancreatic islet cells, which are innervated by the vagus nerve (Loewy *et al.*, 1994). PrP-containing islets were often adjacent, which might suggest infection from a common nidus, such as

innervation by a common nerve branch. Infectivity in the pancreas has been documented previously in natural and experimental scrapie infections (Pattison & Millson, 1960; Ye *et al.*, 1994*b*) and PrP has also been demonstrated in islets from uninfected and scrapie-infected mice (McBride *et al.*, 1992). Alterations in islet function have not been examined, although hamsters infected with the 139H scrapie strain develop obesity and hypoglycaemia/hyperinsulinaemia with extensive pituitary and pancreatic vacuolation (Ye *et al.*, 1994*a*; Ye & Carp, 1996). Pancreatic lesions were localized to islet beta cells, amyloid deposits were not found and scrapie infectivity was extremely low (Ye *et al.*, 1997).

The PrP<sup>CWD</sup> detected in the adrenal medulla could be derived from PrP transport via the splanchnic nerves arising from nerve cell bodies in the intermediolateral column of the spinal cord, which has demonstrable PrP<sup>CWD</sup>. We also demonstrated PrP<sup>CWD</sup> in the pars intermedia and nervosa of the pituitary in CWD-infected deer. Potentially, PrP<sup>CWD</sup> could transit via nerve fibres from the hypothalamus to the pars nervosa, as deer with CWD have abundant PrP<sup>CWD</sup> deposition in the hypothalamus (Spraker *et al.*, 2001). Histological lesions were not evident in either the adrenal or pituitary; however, it is not known whether functional disturbances such as altered hormone synthesis are associated with PrP<sup>CWD</sup> deposition.

In summary, the findings reported here in CWD-infected deer provide circumstantial evidence for: (i) trafficking of PrP<sup>CWD</sup> in the peripheral nerves and (ii) localization of the pathogenic prion protein in the endocrine system of CWD-infected deer. These observations are consistent with assembled findings in other experimental and natural TSEs and may lend insight into the potential pathways of prion trafficking in cervid CWD.

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