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Prions spread via the autonomic nervous system from the gut to the central nervous system in cattle incubating bovine spongiform encephalopathy

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To elucidate the still-unknown pathogenesis of bovine spongiform encephalopathy (BSE), an oral BSE challenge and sequential kill study was carried out on 56 calves. Relevant tissues belonging to the peripheral and central nervous system, as well as to the lymphoreticular tract, from necropsied animals were analysed by highly sensitive immunohistochemistry and immunoblotting techniques to reveal the presence of BSE-associated pathological prion protein (PrP^{Sc}) depositions. Our results demonstrate two routes involving the autonomic nervous system through which BSE prions spread by anterograde pathways from the gastrointestinal tract (GIT) to the central nervous system (CNS): (i) via the coeliac and mesenteric ganglion complex, splanchnic nerves and the lumbal/caudal thoracic spinal cord (representing the sympathetic GIT innervation); and (ii) via the *Nervus vagus* (parasympathetic GIT innervation). The dorsal root ganglia seem to be subsequently affected, so it is likely that BSE prion invasion of the non-autonomic peripheral nervous system (e.g. sciatic nerve) is a secondary retrograde event following prion replication in the CNS. Moreover, BSE-associated PrP^{Sc} was already detected in the brainstem of an animal 24 months post-infection, which is 8 months earlier than reported previously. These findings are important for the understanding of BSE pathogenesis and for the development of new diagnostic strategies for this infectious disease.

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INTRODUCTION

Prion diseases have received considerable attention because of the huge bovine spongiform encephalopathy (BSE) epidemic that affected more than 185 000 clinically and fatally diseased cattle. Roughly 3 million infected animals still in the preclinical state were slaughtered and entered the human food chain in the UK and elsewhere (Donnelly *et al.*, 2002). Transmission of BSE prions to man has eventually caused a variant form of Creutzfeldt–Jakob disease in more than 170 humans primarily in the UK, but also in France, Italy, Japan and elsewhere. As a preventive measure in the European Union, the risk of human BSE exposure is minimized by rapid BSE testing of all cattle over 30 months of age and by the removal of specified risk materials from slaughtered cattle that may possibly contain BSE infectivity in incubating animals. These materials currently include the head and backbone, including the central nervous system (CNS) and spinal cord, of cattle over 12 months of age, and the intestine (independent of age). However, it is presently under discussion in the EU Commission to raise the age limit for head and backbone removal from 12 to 24 months.

During the BSE epidemic, cattle were probably infected by oral uptake of infectious foodstuffs (Wilesmith *et al.*, 1988; Paisley & Hostrup-Pedersen, 2004). However, although several approaches addressing the pathogenesis of BSE have been undertaken, the route and time course of the infectious agent from the gastrointestinal tract (GIT) to the CNS is still unknown. To date, very few data for BSE-incubating preclinical cattle are available.

A feature in the early pathogenesis of orally induced prion diseases (including BSE) is the appearance of PrP^{Sc} in the ileal Peyer's patches, the gut-associated lymphoid tissue (Maignien *et al.*, 1999; Beekes & McBride, 2000; Heggebo *et al.*, 2003; Terry *et al.*, 2003). The subsequent spread of the BSE agent from the gut to the CNS is still an enigma. In experimentally orally challenged cattle, infectivity has been demonstrated convincingly solely in the trigeminal ganglia (Wells *et al.*, 1998) of terminally incubating animals. Moreover, infectivity was detected by using a cattle bioassay in the palatine tonsil of animals killed 10 months post-infection, but not in animals assayed later in the incubation period (Wells *et al.*, 2005).

In contrast to this, PrP^{Sc} and/or infectivity has been detected within the entire lymphoreticular system (LRS) of naturally or experimentally BSE- or scrapie-infected sheep, mice and hamsters (van Keulen *et al.*, 1996; Andreoletti *et al.*, 2000; Aguzzi, 2003; Glatzel *et al.*, 2004; Press *et al.*, 2004), whereas the paucity of BSE prions in these tissues (with the exception of the above-mentioned result) in cattle is striking. Studies in sheep and hamsters infected orally with scrapie indicate an important role of the autonomic nervous system in the spread of the transmissible spongiform encephalopathy (TSE) agent. Two possible neuroanatomical pathways, which use the intramural ganglia of the gut and the coeliac and mesenteric ganglion complex (CMGC) as intervening relay points, are proposed: along the splanchnic nerve to the midthoracic spinal cord and/or along the vagus nerve to the brainstem (van Keulen *et al.*, 2000; McBride *et al.*, 2001). However, to date in cattle, BSE infectivity has only been detected in different parts of the CNS and peripheral nervous system (PNS) in clinical animals (Buschmann & Groschup, 2005; Wells *et al.*, 1998, 2005).

Hence, the aim of the study was to elucidate the infection route and, in particular, the time course of BSE infection in cattle. New insights into the pathogenesis of BSE are important not only for the design of new diagnostic strategies, but also to minimize the risk of human BSE exposure, especially with regard to setting the age limit for the removal of specified risk material of cattle in the EU.

METHODS

Animals. Fifty-six Simmental cross-breed calves aged 4–6 months were challenged orally with BSE using a brainstem-homogenate pool of clinically diseased cattle. Every 4 months, four or five animals were selected randomly and killed. A wide range of tissues was sampled at subsequent necropsy.

Immunohistochemistry (IHC). With some modifications, tissue samples were processed as described previously (Hardt *et al.*, 2000). All tissues samples were fixed in 4 % buffered formalin, treated for 1 h with 98 % formic acid, rinsed for 40 min in tap water, embedded in paraffin, sectioned at 3–4 μm and stained with haematoxylin and eosin.

The avidin–biotin complex (ABC) method was used for PrP-IHC. The paraffin-wax tissue sections were mounted on Superfrost Plus slides (Menzel–Gläser) and rehydrated. The subsequent pretreatment included incubation of the slides in 98 % formic acid for 15 min, a 5 min rinse in tap water, inhibition of the endogenous peroxidase activity with 3 % H_2O_2 (Merck) in methanol for 30 min, followed by 15 min digestion with proteinase K (4 μg ml^{-1}; Boehringer Mannheim) at 37 °C. The primary monoclonal antibodies (mAbs) were applied at a dilution of 1 : 1800 for mAb 12F10 (Cayman Chemical) and 1 : 250 for mAb L42 (Harmeyer *et al.*, 1998) in goat serum and incubated at 4 °C overnight. Negative-control sections were treated with a mAb against GP_5 of *Porcine respiratory and reproductive syndrome virus* (Weiland *et al.*, 1999). As a secondary (link) antibody, biotinylated goat anti-mouse antiserum (Vector Laboratories) was incubated on the sections in a 1 : 200 dilution for 30 min at room temperature. Immunodetection was amplified by using Vector ABC-elite avidin–horseradish peroxidase–biotin complex (Vector Laboratories). The slides were finally developed in diaminobenzidine tetrahydrochloride (Fluka Feinchemikalien) and counterstained with Mayer's haematoxylin. All sections were examined by light microscopy.

Western blot (WB) analysis. Selected tissue samples were investigated for the accumulation of PrP^{Sc} by Western blotting using phosphotungstic acid precipitations (PTA-WB), which were carried out according to a protocol established previously (Wadsworth *et al.*, 2001; Glatzel *et al.*, 2004) with some modifications described elsewhere (Gretzschel *et al.*, 2005).

Rapid tests. To confirm the IHC results at the level of the obex, adjacent brainstem material from the cranial and caudal medulla was examined by using the IDEXX HerdChek rapid test following the manufacturer's instructions.

RESULTS

Fifty-six Simmental cross-breed calves at 4 months of age were challenged orally with a macerate of 225 BSE-positive brainstems [100 g in a 50 % (w/v) mash containing 5 % sucrose per animal]. Another 18 animals received a non-infectious cattle brainstem homogenate to serve as negative controls. The infectivity load in the BSE brainstem homogenate used for the cattle infection study was $10^{6.1}$ ID_{50} $(g\ tissue)^{-1}$ as determined by end-point titration in Tgbov XV mice (Spearman, 1908; Kärber, 1931; Buschmann & Groschup, 2005) (data not shown). The cattle were housed in a special TSE infection facility and were assessed clinically every 2 months. Every 4 months, four or five randomly selected animals were euthanized and necropsied under TSE sterile conditions and more than 150 tissue and bodily fluid samples were collected from each animal.

To determine the location and earliest time point of a PrP^{Sc} deposition in the CNS or PNS, as well as in the LRS and viscera, the most relevant tissue samples (Table 1), i.e. brainstem at the level of the obex and distal ileum, were analysed by IHC. Here, results from two clinically normal animals that were killed after 24 months (cow A) and 28 months (cow B) and in which a PrP^{Sc} deposition at the obex was detectable at the earliest time point post-oral challenge are described. A full report covering all other animals will be published separately.

In the two animals with the earliest PrP^{Sc} deposition in the brainstem, all relevant tissues on the potential infection route for the BSE prions from the distal ileum to the CNS were examined by IHC and/or PTA-WB. These were the GIT and the associated lymph nodes, tonsils, retropharyngeal lymph nodes and spleen, as well as large parts of the sympathetic and parasympathetic nervous system, nerve fibres and ganglia (Table 1).

Both animals showed, like many other cows in the herd, moderate to severe eosinophilic enteritis, predominantly in the small intestine. In cow A, this was accompanied by moderate eosinophilic cholangitis and mild bile ductule proliferation. These alterations were probably due to mild coccidiosis as shown by light microscopy and in the parasitological examination. No other histopathological alterations were observed in the CNS or elsewhere.

Table 1. Tissue samples investigated by immunohistochemistry and PTA-immunoblot for detection of BSE-specific PrP^{Sc}

Tissue	Cow A		Cow B	
	IHC	No. of positive samples/total no. examined	IHC	No. of positive samples/total no. examined
Central nervous system				
Brain				
Obex	+	1/1	+	1/1
Medulla oblongata	+*	1/1	−	0/3
Pons	+	1/1	ND	
Midbrain	−	1/1	ND	
Spinal cord				
Cervical:				
C2	+	1/2	−	0/3
C4	+	1/1	−	0/3
C6	+	2/3	−	0/3
Thoracic:				
T1	+	1/2	−	0/3
T3	+	2/2	−	0/3
T5	+	2/2	−	0/3
T7	+	1/1	−	0/3
T10	+	2/3	−	0/3
T12	−	0/3	−	0/3
Dorsal root ganglia (T1–8)	−	0/5	−	0/3
Lumbal:				
L1	−	0/3	−	0/2
L3	+	1/1	−	0/2
L6	−	0/3	−	0/2
Cauda equina	−	0/3	−	0/2
Peripheral nervous system				
Ganglion nodosum	−	0/2	−	0/4
Ganglion cervicale craniale	−	0/5	−	0/1
Ganglion trigeminale	−	0/4	−	0/3
Ganglion aorticorenalis	−	0/4	−	0/2
Ganglion coeliacum	+	2/5	NA	
Ganglion mesentericum caudale	+	1/4	−	0/5
Ganglion stellatum	−	0/4	−	0/2
Ganglion vertebrale cervicale caudale	−	0/3	−	0/2
Ganglion thoracicum vertebrae	−	0/5	−	0/1
Nervus facialis	−	0/5	ND	
Nervus opticus	−	0/5	ND	
Nervus phrenicus	−	0/3	−	0/2
Nervus splanchnicus	−	0/5	−	0/3
Nervus ischiadicus/Plexus brachialis	−	0/6	ND	
Nervus vagus (several locations)	−	0/20	−	0/8
Plexus pelvinus	−	0/5	−	0/2
Lymphoid tissue				
Peyer's patches				
Ileum	+	3/3 (12/52 follicles)	−	0/3 (44 follicles)
Ileocaecal plica	−*	0/3 (0/173 follicles)	−	0/3 (0/62 follicles)
Jejunum	−	0/6 (0/63 follicles)	−	0/6 (0/92 follicles)
Colon	−	0/6 (0/72 follicles)	−	0/3 (no follicles)
Rectum	−	0/3 (0/204 follicles)	−	0/3 (0/106 follicles)
Gut-associated lymphoid tissue†	−	0/15	−	0/7
Lymphonodi retropharyngeales	−	0/3	−	0/2
Lymphonodi iliaci mediales	−	0/3	ND	
Spleen	−	0/3	−	0/1
Bone marrow (femur)	−	0/3	ND	

Table 1. cont.

Tissue	Cow A		Cow B	
	IHC	No. of positive samples/total no. examined	IHC	No. of positive samples/total no. examined
Gastrointestinal tract				
Oral mucosa (including pharynx)	–	0/4	–	0/2
Tongue	–	0/1	–	0/1
Oesophagus	–	0/1	–	0/1
Forestomach, abomasum	–	0/5	–	0/3
Small intestine	–	0/12	–	0/8
Large intestine	–	0/12	–	0/15
Rectum	–	0/3	–	0/2
Viscera				
Liver	–	0/1	–	0/1
Kidney	–	0/1	–	0/1
Heart	–	0/1	–	0/1
Lung	–	0/1	–	0/1

ND, Not done; NA, not available.
*Tissue with additional PrP^{Sc} detection by using PTA-immunoblot.
†Includes tonsil and *Lymphonodi jejunales*, *ileales*, *lienales* and *colici*.

Cow A

In the intestine, IHC immunolabelling was confined to the follicles of the Peyer's patches of the distal ileum. A predominantly globular reaction pattern was observed in the cytoplasm of large mononuclear cells in the light central zone of 12 of 52 follicles examined (Fig. 1a). Additionally, very fine granular immunolabelling was found within single follicles. PrP^{Sc} was also found in the ileocaecal plica by PTA-WB, but nowhere else in the GIT (Table 1). BSE-specific PrP^{Sc} was not detected in the enteric nervous system (ENS) (myenteric or submucosal plexi) of the distal ileum or elsewhere.

The presumed next BSE-PrP^{Sc}-positive site on the assumed transmission route to the CNS was the CMGC, in which single neuronal cells presented sparse, but clear, perineuronal labelling and weak intraneuronal labelling (Fig. 1b). Accumulated PrP^{Sc} was also seen in satellite cells. Moreover, a similar reaction pattern was observed in the *Ganglion mesentericum caudale* (GMC). Representing the next step in the autonomic nervous system, almost all

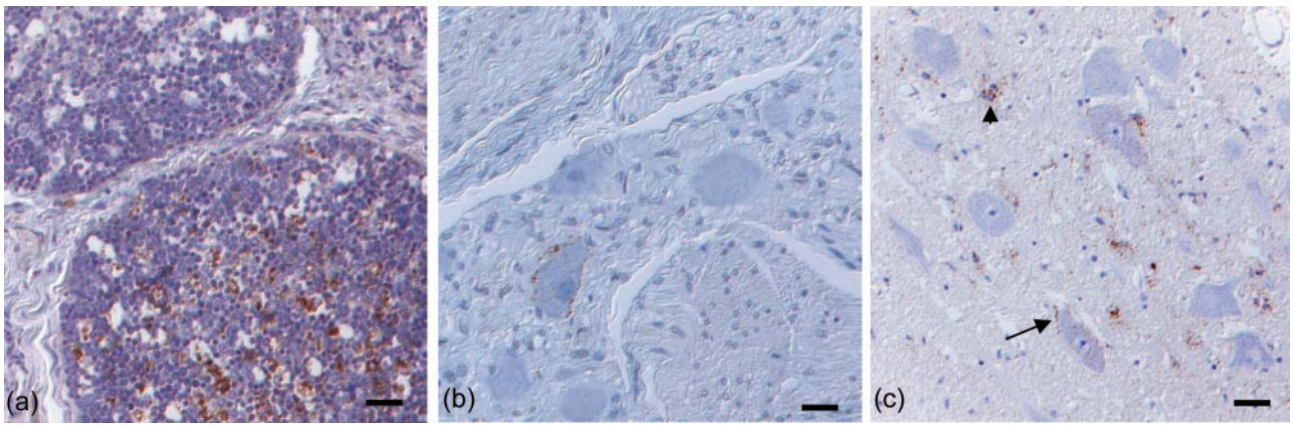

Fig. 1. Distinct PrP^{Sc} immunolabelling in different tissues of a cow at 24 months post-infection. (a) Lymphoid follicle of the ileal Peyer's patches with moderate immunoreactivity in the cytoplasm of large mononuclear cells. (b) Single cell in the CMGC with perineuronal deposits of PrP^{Sc}. (c) Mild intraneuronal, perineuronal (arrow), intraglial (arrowhead) and granular PrP^{Sc} immunostaining in the obex region (DMNV). Immunohistochemistry, PrP mAb 12F10, Nomarski interference contrast. Bars, 25 μm.

segments of the spinal cord, with the exception of the caudal lumbal region and the *Cauda equina*, were shown to contain BSE-PrP^{Sc} by IHC and/or PTA-WB (Table 1). Again, only single neuronal cells in the *Substantia intermedia centralis* and *lateralis* displayed a mild intraneuronal PrP^{Sc} accumulation and, in some sections, even a weak linear staining pattern. No specific PrP^{Sc} staining was detected in ependymal cells or in the dorsal root ganglia of the thoracic spinal cord.

In the brain, PrP^{Sc} deposits were confined, bilaterally symmetrically, to the dorsal motor nucleus of the vagus (DMNV) at the level of the obex and were characterized by a diffuse distribution with slightly stronger immunolabelling in the medial parts of the DMNV. The mild reaction patterns were intraneuronal, perineuronal and linear when adjacent to neuronal cells, as well as intraglial and diffuse granular in the neuropil (Fig. 1c). Additionally, a weak intraneuronal staining reaction of single cells was detectable in the *Medulla oblongata*, confirmed by the results of PTA-immunoblotting (Fig. 2), and in the pons region, but not in the midbrain (Table 1).

A moderate positive reaction was observed in the cranial (OD 0.669) and caudal (OD 0.222; cut-off 0.221) parts of the *Medulla oblongata* (both adjacent to the region of the obex) by using the IDEXX HerdChek BSE rapid test.

Cow B

In the entire GIT, neither the lymphoid tissues of the gut nor the compartments of the ENS showed detectable amounts of PrP^{Sc} (Table 1). Moreover, PrP^{Sc} was not detected in the autonomic nervous system or the entire spinal cord of this animal. However, mild perineuronal immunostaining of single cells was observed at the ventrolateral margin of the DMNV at the level of the obex. No immunoreactivity was found in the adjacent parts of the *Medulla oblongata*.

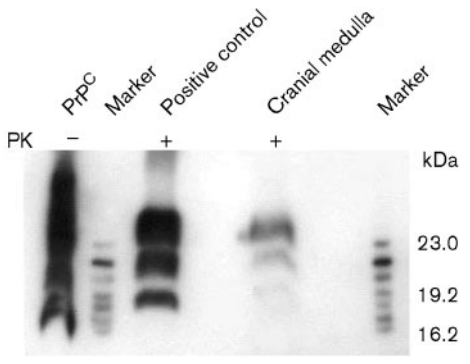

Fig. 2. PTA-immunoblot after proteinase K (PK) digestion showing PrP^{Sc} reactivity in the cranial parts of the medulla oblongata (adjacent to the region of the obex) using mAb L42.

Interestingly, a negative result in the rapid test was obtained for the cranial and caudal parts of the *Medulla oblongata* (both adjacent to the level of the obex) of this animal.

DISCUSSION

The data presented here demonstrate for the first time that BSE prions can reach the brain in as little as 24 months after a massive oral challenge. In an earlier BSE pathogenesis study, which was of similar design, the first PrP^{Sc} deposition was observed in the brainstem 32 months after exposure. Moreover, a brainstem pool of a single cow sacrificed 26 months post-challenge did not contain infectivity when bioassayed in cattle (Wells, 2003; Wells *et al.*, 2005). These differences may be due to a considerable biological variation in the timing of the pathogenesis of BSE in cattle in individual animals. Although unlikely, the influence of the breed of cattle examined (Holstein–Friesian cattle used in UK studies versus Simmental cross-breed calves used here) cannot be ruled out. Similarly, it is conceivable that the gut-associated inflammatory processes, observed in both cows, may have had an effect on the progress of disease, a phenomenon that has already been shown in mice (Thackray *et al.*, 2003).

The BSE-challenged cows described here were clearly at the threshold of the earliest detection of PrP^{Sc} by currently available IHC and PTA-WB methods. The IHC examination was carried out with extreme scrutiny in order to discover even minor traces of PrP^{Sc} staining that may not be detected under routine conditions. There was just a mild immunostaining of single neuronal cells in the brainstem of cow A. Moreover, it should be emphasized that, in cow B, the IHC reaction was restricted to the DMNV at the obex. This limited IHC staining (as confirmed by analysing different parts of the brainstem, including the midbrain of cow A) indicates that no spread of BSE prions to other brain areas had taken place before this early time point post-infection.

In accordance with previous studies, an accumulation of PrP^{Sc} was observed in cow A in the Peyer's patches of the distal ileum, mostly in tingible body macrophages and, to a lesser extent, in follicular dendritic cells, which are characterized by a fine reticular staining pattern (Andreoletti *et al.* 2000). However, PrP^{Sc} deposition was not detected in the ENS of the entire GIT. Both results are in contrast to the reported distribution of the scrapie agent in sheep (Heggebo *et al.*, 2003), where PrP^{Sc} was consistently found in the ENS when there was also abundant PrP^{Sc} deposition in the gut-associated lymphoid tissue. To verify the sensitivity of our IHC technique, intestine samples from scrapie-diseased sheep were also examined; clear and strong PrP^{Sc} immunolabelling was observed in the ENS as expected (data not shown). Therefore, two possible routes may be considered for BSE prion spread in cattle in the GIT: (i) a direct transmission of the BSE agent from the

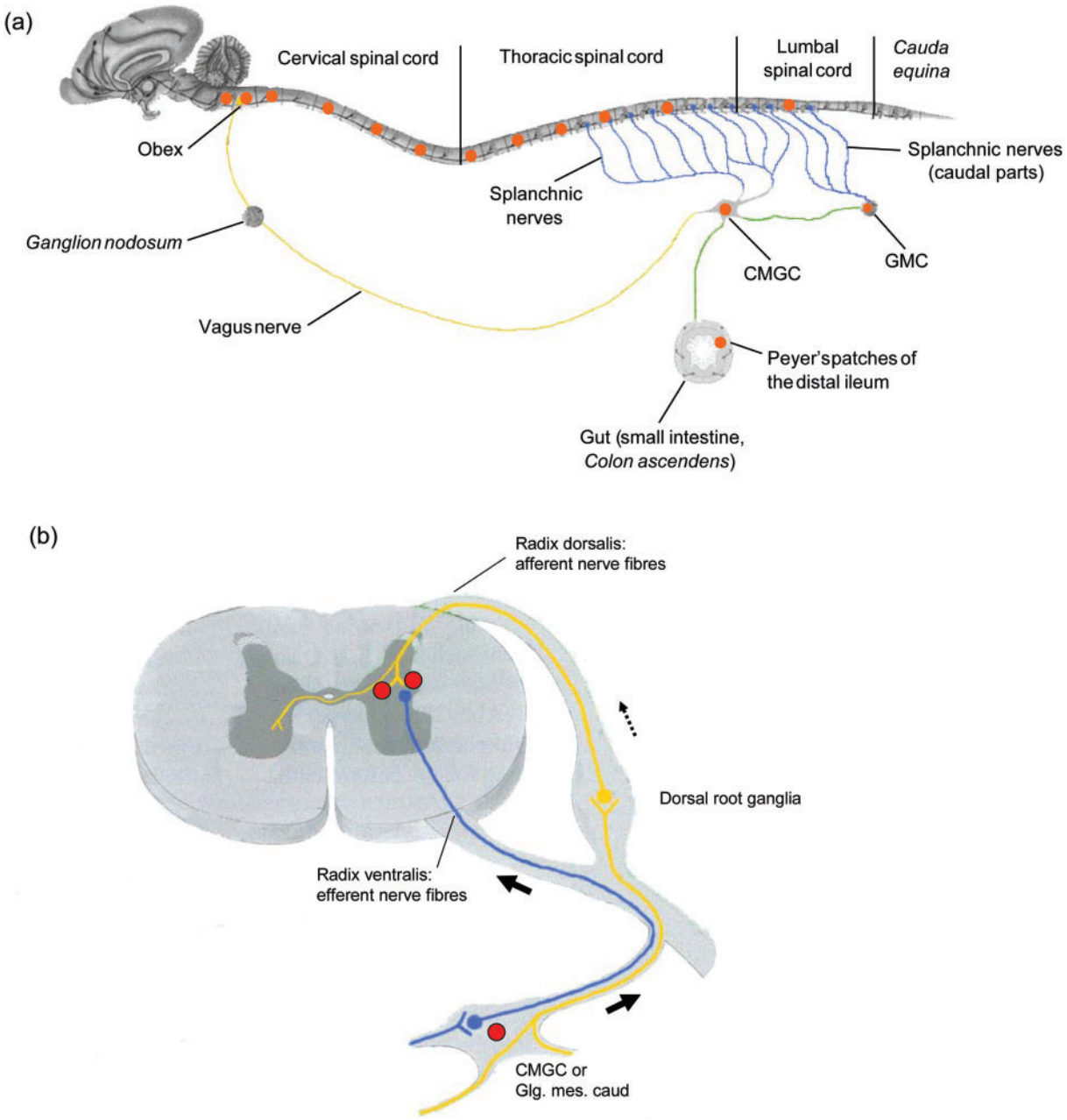

Fig. 3. Schematic overview. Regions with distinct PrP^{Sc} deposition are drawn in red. (a) The most likely routes of BSE prions from the gut into the brain via the autonomic nervous system. Blue, sympathetic nerve fibres; yellow, parasympathetic nerve fibres of the vagus nerve; green, mixed autonomic fibres. It is important to know that as a result of the crossing over in the CMGC, there is no localized projection zone for the splanchnic territory in the spinal cord. (b) BSE prion spread from the coeliac and mesenteric ganglion complex to the spinal cord. Blue, efferent nerve fibres; yellow, afferent nerve fibres. The thick arrows indicate the most probable infectivity routes into the spinal cord.

gut lumen to the network of nerve fibres in the submucosa (Balemba *et al.*, 1999) without further replication and subsequent accumulation in the neurons of the ENS and in the lymphoid tissue. The PrP^{Sc} accumulation in the Peyer's patches reported here and elsewhere (Terry *et al.*, 2003) could be due to tingible body macrophages involved in PrP^{Sc} clearance. (ii) An alternative explanation would be locally restricted uptake of BSE prions in the ileum and subsequent replication in the local follicles. This hypothesis is supported by the small amount of randomly distributed follicles that were positive in cow A and the lack of detectable amounts of PrP^{Sc} in cow B (tissue sections as

thin as 3 μm may not be representative of the complete ileum when only a few follicles are affected).

Both hypotheses are supported by the data reported by Terry *et al.* (2003), which show sparse immunolabelling only in neurons of the ENS in two animals (38 and 40 months after exposure). Apart from the local Peyer's patch invasion, the lymphoreticular tract seems not to be affected in cattle, as the adjacent *Lymphonodi ileales* (similar to the *Lnn. jejunales*, *lienales* and *colici*) were free of any detectable PrP^{Sc}.

The detection of PrP^{Sc} reported here in the DMNV, CMGC and GMG, as well as in the *Substantia intermedia centralis* and *lateralis* of the spinal cord of BSE-infected cattle, suggests two routes that BSE prions may take to the brain (Fig. 3). Both CMGC and GMC ganglia contain sympathetic and parasympathetic nerve fibres. Therefore, the most likely route by far follows the efferent sympathetic fibres of the *Nervi splanchnici majores* and *minores*, which contain nerve fibres crossing over in the CMGC, to the thoracic and/or lumbal spinal cord (T6–L2). It must be emphasized that both parts of the spinal cord can innervate the same part of the intestine (Fig. 3a). The importance of this pathway is also supported by immunolabelling of the sympathetic (in parts, splanchnici-associated) pre-ganglionic neuronal cells in the *Substantia intermedia centralis* and *lateralis* of the spinal cord. However, other than in the hamster scrapie model, in which an initial PrP^{Sc} accumulation in the mid-thoracic region and delayed deposition in other areas of the spinal cord (Beekes *et al.*, 1996; Baldauf *et al.*, 1997) were observed, all thoracic spinal cord segments, with the exception of the most caudal part (Th12), were affected evenly in the BSE-infected cow A, indicating an almost-simultaneous prion invasion through the *Nn. splanchnici.* However, this pattern could also have arisen from a focal invasion followed by a subsequent retro- and anterograde spread in the spinal cord. Moreover, the lack of involvement of the thoracic dorsal root ganglia, which contain the afferent neurons, also indicates the spread of PrP^{Sc} along the efferent nerve fibres via the *Radix ventralis* directly to the pre-ganglionic neuronal cells (Fig. 3b).

Furthermore, a retrograde spread from the CMGC to the GMC is probable, as PrP^{Sc} was not detected in the large intestine, which is innervated by this caudal mesenteric ganglion. In addition, the subsequent infection along the *N. splanchnici lumbales*, which crossover in the GMC, would explain the sporadic appearance of PrP^{Sc} immunostaining in the mid-lumbal spinal cord (L3) (see Fig. 3).

The second possibility for the spread of BSE prions from the CMGC to the brain follows the parasympathetic nerve fibres of the vagus nerve, although it was not possible to demonstrate PrP^{Sc} accumulation in this nerve itself. This result supports the hypothesis of McBride *et al.* (2001) that PrP^{Sc} is in transit in nerve fibres rather than actively replicated. However, both cows showed a clear DMNV-associated PrP^{Sc} immunostaining of singleton neuronal cells at the level of the obex and cow B displayed minute immunostaining at this site without any other neuronal nuclei involved. This pattern is indicative of anterograde spread along the parasympathetic nerve fibres, which has already been described in hamsters infected orally with scrapie (McBride & Beekes, 1999; McBride *et al.*, 2001). Moreover, in cow B, there was no PrP^{Sc} immunoreactivity detectable by IHC or PTA-immunoblot in the spinal cord. This result also indicates early BSE prion transmission along the vagus nerve.

In conclusion, results obtained here clearly suggest a neuronal rather than a lymphoreticular progression of BSE prions to the brain. Moreover, a simultaneous spread in the early pathogenesis of BSE is postulated: along the parasympathetic nerve fibres of the vagus nerve to the brain and via the sympathetic splanchnic nerves to the spinal cord and subsequently to the brain. The relatively short period of 24 months for the appearance of PrP^{Sc} deposition in the brain is of particular interest. This finding, although produced after challenge with a massive dose of BSE infectivity, which has been obtained 8 months earlier than described in similar experiments, should be taken into consideration during the discussion on the age limit for removal of brain and spinal cord as specified risk material of cattle in the EU.

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