Short Communication

Enteroglial and neuronal involvement without apparent neuron loss in ileal enteric nervous system plexuses from scrapie-affected sheep

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The enteric nervous system (ENS) probably plays a dominant role in sheep scrapie pathogenesis, but little is known about the cell types involved. We investigated the ileal myenteric and submucosal plexuses of four naturally and four orally experimentally scrapie-affected ARQ/ARQ Sarda sheep, as well as those of 12 healthy-control Sarda sheep carrying different PrP genotypes. All scrapie-affected animals, euthanized at clinical-disease end stage, showed PrPd deposition within enteric glial cells (EGCs) and calbindin-immunoreactive (CALB-IR) and neuronal nitric oxide synthase (nNOS)-IR neurons. Whole-mount investigations revealed no significant differences between the densities of total, CALB-IR and nNOS-IR neurons in scrapie-affected versus healthy sheep, irrespective of PrP genotype. Our results suggest that EGCs and CALB-IR and nNOS-IR neurons are probably involved in the pathogenesis of natural and oral experimental sheep scrapie. Furthermore, the infectious agent may be less pathogenic towards ENS neurons than it is towards central nervous system neurons.

Sheep scrapie is the ‘prototype’ of transmissible spongiform encephalopathies (TSEs) or prion diseases, a group of neurodegenerative disorders affecting humans and animals. The key pathogenetic event in TSEs is the accumulation within the central nervous system (CNS) and peripheral tissues of an abnormal isoform (disease-specific PrP, PrPd) of the host-encoded cellular prion protein, PrPC (Aguzzi & Polymenidou, 2004).

Genotype of the host is known to modulate susceptibility/resistance to TSEs, which is dependent on polymorphisms throughout the PrP gene (PRNP) (Prusiner, 1998). In sheep, PRNP codons 136, 154 and 171 are of greatest importance in modulating susceptibility/resistance to scrapie (Goldmann et al., 1994). In Suffolk and Sarda breeds (Westaway et al., 1994; Vaccari et al., 2001), codon 171 plays a major role in disease-susceptibility control, whereas in Cheviot sheep, the additional influence of codon 136 has been reported (Goldmann et al., 1994).

The gastrointestinal tract is probably the natural prion entry site, with the enteric nervous system (ENS) playing, along with palatine tonsils and Peyer’s patches (PPs), a crucial role in the early pathogenesis of animal and human TSEs (Mabbott & MacPherson, 2006). It has been suggested that ENS plexuses could act as the initial site...
of neuroinvasion for prions, which may subsequently gain access to the CNS through sympathetic and parasympathetic efferent fibres (Aguzzi & Heikenwalder, 2006).

Nevertheless, no definitive information is available on the cellular basis for prion transmigration from gut to ENS plexuses, nor on the ENS cell types colonized by TSE agents. Likewise, the morphofunctional changes affecting the ENs cell populations during infection are unknown, despite the existence of studies addressing CNS neuron damage and targeting on behalf of TSE agents (Guentchev et al., 1999).

This study was aimed at characterizing the ENS cells that are targeted during natural and oral experimental scrapie infection in Sarda sheep. Major emphasis was placed upon enteric glial cells (EGCs) and two neuron populations expressing calbindin (CALB) and neuronal nitric oxide synthase (nNOS), respectively. In sheep, CALB-immunoreactive (IR) cells account for 20–25 % of myenteric plexus (MP) and 65–75 % of submucosal plexus (SMP) neurons, most of them corresponding to cholinergic Dogiel type II neurons and probably acting as intrinsic primary afferent neurons (Chiocchetti et al., 2004, 2006). Enteric nNOS-IR cells, accounting for 31–36 % of MP and 22–24 % of SMP neurons (Lalatta-Costerbosa et al., 2007), correspond to Dogiel type I cells, probably acting as inhibitory motor-neurons (Pfannkuche et al., 2002).

Another objective was to evaluate whether neuron loss occurs within ileal ENS plexuses from naturally and orally experimentally scrapie-affected Sarda sheep. Indeed, whilst CNS neuron loss is constantly observed in TSEs (Pocchiari, 1994), no data concerning ENS are available.

Ileal MPs and SMPs were obtained from 20 Sarda sheep carrying different PrP genotypes, which were characterized as reported elsewhere (Vaccari et al., 2001). Twelve sheep (three ARQ/ARQ, seven ARR/ARQ and two ARR/ARR animals), aged 2–4 years and originating from a scrapie-free flock, acted as healthy controls and were slaughtered according to standard procedures, with tissue samples (cerebral obex, palatine tonsils and distal ileum) being collected from them. The remaining animals included four ARQ/ARQ naturally scrapie-affected and four additional ARQ/ARQ sheep that had been dosed orally at 20 days of age with 25 ml 20 % scrapie brain homogenate. These animals were euthanized, in accordance with approved protocols, at the terminal stage of disease (between 2 and 5 years), with the same tissues being collected. The diagnosis of scrapie was confirmed or excluded by immunohistochemistry (IHC) and Western blot (WB) analysis, which was carried out on the obex, tonsil and distal ileum, in agreement with published protocols (Ligios et al., 2006).

Ileal samples were fixed in 10 % neutral-buffered formalin, embedded in paraffin and cut into 5 μm thick sections, both transverse and tangential, which were placed on glass slides coated with (3-aminopropyl)triethoxy-silane (Sigma-Aldrich).

PrPd IHC was performed with a mouse monoclonal antibody (mAb) (F99/97.6.1; VMRD, Inc.). A pre-treatment protocol was applied to abolish PrPc immunoreactivity without damaging other epitopes and preserving tissue morphology (Kovács et al., 2005). Sections were rinsed in 96 % formic acid for 2 min at room temperature and autoclaved at 100 °C for 20 min in 0.01 M citrate buffer (pH 6.0). Antibody binding was detected by using a biotin–avidin method and immune reactions were visualized by 3,3'-diaminobenzidine (DAB) chromogen solution (both from Vector Laboratories, Inc.). Control sections included obex, tonsil and distal ileum from scrapie-infected and uninfected Sarda sheep. Additional negative controls were set up by omitting the primary antibody.

Double-labelling indirect immunofluorescence (DLIIF) was carried out with a panel of commercial mAbs and polyclonal (Po)Abs against PrPc (F99/97.6.1 mAb), glial fibrillary acidic protein, an EGC marker (anti-GFAP PoAb), neuron-specific enolase, a neuron cell marker (anti-NSE PoAb), nNOS and CALB (both PoAbs) (see Supplementary Table S1, available in JGV Online, for details). The same protocol to abolish PrPc immunoreactivity was employed in DLIIF and then two primary antibodies were added to sections and incubated overnight at room temperature. PrPd was detected by an enhanced biotin–avidin protocol (Vector Laboratories, Inc.) and sections were incubated with goat anti-mouse and anti-rabbit biotinylated secondary antibodies for 30 min. Immune reactions were visualized by Texas red avidin DCS (D cell sorter) and fluorescein avidin DCS fluorochromes (Vector Laboratories, Inc.). Control sections included obex, tonsil and distal ileum from scrapie-infected and uninfected Sarda sheep. Additional negative controls were set up by omitting primary antibodies. Sections were examined under a Nikon Eclipse 800 microscope equipped with fluorescence and images were collected with a Nikon DXM 1200 digital camera. Colocalization analyses were performed by ImageJ software (National Institutes of Health).

To estimate the density (cells mm⁻²) of total (HuC/D-IR), nNOS-IR and CALB-IR neurons within ileal MPs, whole-mount (WM) preparations were made. We utilized a protocol originally developed in the rat (Phillips et al., 2004), adapting it to the sheep ileum, where MPs show a more variable and less geometric texture (Gabella, 1987) and MP neurons are often gathered in large ganglia exhibiting a polygonal or ring-like morphology, with no apparent directionality (Lalatta-Costerbosa et al., 2007). Beginning 2 cm oral to the ileocaecal junction, we collected from each animal 8–10 cm long segments, which were cut along the mesenteric border, pinned on balsa board and fixed in 2 % paraformaldehyde plus 0.2 % picric acid in PBS (pH 7.0) at 4 °C overnight, before being washed three times in DMSO (10 min each) and stored at 4 °C in PBS containing sodium azide (0.1 %). Segments were subdivided into six transverse columns, with a 1×1 cm
sample being collected from each column at different levels, to achieve an adequate representation of the entire circumference (from anti-mesenteric to mesenteric border) and length of every segment. WM preparations were obtained by removing the mucosa, submucosa and circular muscle layer from each sample. For DLIIF investigations, MPs were incubated in 10% normal goat serum in PBS containing 1% Triton X-100 for 30 min at room temperature. An anti-HuC/D mAb was used as pan-neuron marker (Chiocchetti et al., 2004; Phillips et al., 2004), whereas CALB-IR and nNOS-IR neurons were detected with specific PoAbs (see Supplementary Table S1, available in JGV Online, for details). Samples were challenged simultaneously with two primary antibodies for 40 h at 4°C, washed three times in PBS (10 min each) and finally incubated with goat anti-mouse and anti-rabbit secondary antibodies conjugated with Alexa 594 (Molecular Probes) and fluorescein isothiocyanate (FITC) 492 (Calbiochem) fluorochromes, respectively.

For each of the six samples, the number of total (HuC/D-IR), CALB-IR and nNOS-IR cells was counted in 12 microscope fields (each field, 0.28 mm²), which had previously been determined by means of two orthogonal coordinates taken from a table of random numbers and measured on the movable stage of the microscope. Therefore, for each stain per plexus per animal, a total area of 10.08 mm² was evaluated. To avoid missing small-sized or faintly stained cells, MP neurons were counted by two independent investigators blind to the experimental conditions at ×40 magnification with a Zeiss Axioplan microscope equipped with appropriate filters discriminating the fluorochromes used. Conventionally, neurons intersected by the upper and lower fields’ hemicircumferences were, respectively, disregarded and considered (De Souza et al., 1993).

All quantitative data collected, not grouped for control/infected status and PrP genotype, were tested for normality with a Shapiro–Wilk W test. As the Shapiro–Wilk W test did not demonstrate a normal distribution, comparisons among healthy controls carrying different PrP genotypes and between controls – irrespective of PrP genotype – and scrapie-affected sheep were evaluated with Spearman’s rank correlation test. Statistical analyses were performed by using CSS software (StatSoft) and a conventional 5% level was used to define statistical significance.

All scrapie-affected sheep showed IHC evidence of PrPd deposition in ileal MPs, SMPs and PPs, as well as in tonsils and obex. WB analysis confirmed the above results. PrPd accumulation was more prominent at the MP level, with a ‘pepper granule-like’ pattern strongly compatible with EGCs. A simultaneous involvement of neuronal perikarya was observed. No specific immunolabelling was present in negative-control sections. By DLIIF, PrPd deposition

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**Fig. 1.** Naturally scrapie-affected Sarda sheep. Distal ileum (tangential section). (a–c) Specific anti-CALB (a) and anti-PrPd (b) immunostaining is shown within an SMP neuron. Colocalization of the two signals is shown in white (c). (d–f) Specific anti-nNOS (d) and anti-PrPd (e) immunolabelling is observed within two neuronal perikarya and nerve fibres from an MP. Colocalization of the two signals is shown in white (f). DLIIF with anti-CALB, anti-nNOS and anti-PrP antibodies was performed. Fluorescein avidin DCS (green) and Texas red avidin DCS (red) were used as fluorochromes. Colocalization analyses were carried out by means of ImageJ software. Bars, 50 μm.
involved EGCs and neurons (NSE-IR cells) of MPs and SMPs from the eight scrapie-affected sheep. Both CALB-IR and nNOS-IR neurons (Fig. 1) harboured discrete, granular PrPd aggregates in cell bodies and fibres, with no apparent quantitative differences between these cell populations. In contrast, EGCs exhibited more prominent and diffuse PrPd accumulations (Fig. 2). Negative-control sections showed no evidence of PrPd deposition.

No statistically significant differences in the density of total, CALB-IR and nNOS-IR neurons were observed within ileal MPs among the different PrP genotypes of the 12 control sheep. Also, no statistically significant differences were found between the eight scrapie-affected and the 12 control sheep – irrespective of PrP genotype – when densities of total, CALB-IR and nNOS-IR neurons were compared (Fig. 3).

This study supports the assumption that ileal ENS plexuses are involved in the pathogenesis of both natural and oral experimental scrapie infection in sheep. However, as all scrapie-affected animals were investigated at clinical-disease end stage, we cannot exclude the possibility that ENS plexuses have also been colonized by centrifugal spread, from CNS to gut. By IHC, granular PrPd deposits were detected within ileal MPs and SMPs from scrapie-affected sheep. As reported previously (Heggebø et al., 2003; Jeffrey et al., 2006), the accumulation pattern was mainly consistent with EGCs, although neuronal perikarya and fibres also showed evidence of PrPd deposition.

DLIIF and colocalization analyses confirmed PrPd accumulation in EGCs, CALB-IR and nNOS-IR neurons. Neurons of ENS plexuses express PrP$^C$ (Heggebø et al., 2000; Shmakov et al., 2000) and may show PrPd deposition in different TSEs (Andrèoletti et al., 2000; McBride et al., 2001; Sigurdson et al., 2001; van Keulen et al., 2002). However, PrPd was more consistent detectable within EGCs, which in humans were also shown to express PrP$^C$ (Shmakov et al., 2000), a crucial prerequisite for PrPd deposition and for the progression of infection (Blattler et al., 1997). EGCs are the morphofunctional equivalent of CNS astrocytes (Cabarrocas et al., 2003), which harbour PrPd earlier than CNS neurons (Diedrich et al., 1991) and probably play a role in prion-induced neuron damage (Raebet al., 1997; Jeffrey et al., 2004).

We found no differences in the density of total, CALB-IR and nNOS-IR neurons among control sheep with different PRNP polymorphisms. This suggests that the host’s PrP genotype neither affects the density and/or the neurochemical code of ileal MP neurons in Sarda sheep, as was reported previously for nNOS-IR cells (Lalatta-Costerbosa et al., 2007), nor does it modulate scrapie susceptibility/resistance by influencing MP features. As no differences were detected between scrapie-affected and scrapie-negative sheep, our data also suggest that ENS neurons may

![Fig. 2. Naturally scrapie-affected Sarda sheep. Distal ileum (tangential section). Specific anti-GFAP (a) and anti-PrPd (b) immunostaining is shown within the EGCs of a large MP. Colocalization of the two signals is shown in white (c). Fluorescein avidin DCS (green) and Texas red avidin DCS (red) were used as fluorochromes. Colocalization analyses were carried out by means of ImageJ software. Bars, 50 μm.](image)

![Fig. 3. Mean densities of total (HuC/D-IR), CALB-IR and nNOS-IR neurons within ileal MPs from the 12 control Sarda sheep carrying different PrP genotypes and from the eight ARQ/ARQ (naturally and experimentally) scrapie-affected sheep. No statistically significant differences ($P \leq 0.05$) were detected between healthy-control (filled bars) and scrapie-affected (empty bars) animals (Spearman’s rank correlation test).](image)
interact with the infectious agent in a unique way. Indeed, it is widely accepted that CNS neuron loss represents a dominant feature in TSEs (Pocchiari, 1994). Evidence exists that microglial cells are involved in the pathogenesis of TSE-associated neuron damage at brain level (Peyrin et al., 1999; Pasquali et al., 2006; Priller et al., 2006), but ENS plexuses do not physiologically host a microglial component, a plausible explanation for the apparent lack of neuron loss in our scrapie-affected sheep. We cannot, however, exclude the possibility that qualitative changes were still affecting ENS neurons.

In conclusion, this study showed that EGCs and neurons, particularly CALB-IR and nNOS-IR cells, within ileal ENS plexuses accumulate PrPd during natural and oral experimental scrapie infection in Sarda sheep, without apparent neuron loss. This intriguing observation warrants further study on the complex biological interplay between prions and neurons.

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**References**


