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ORIGINAL PAPER

Myenteric neurons of the ileum that express somatostatin are a target of prion neuroinvasion in an alimentary model of sheep scrapie

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Abstract Neuroinvasion of the enteric nervous system by prions is an important step in dissemination to the brain, yet very little is known about the basic process of enteric neuroinvasion. Using an alimentary model of neonatal disease transmission, neuroinvasion by scrapie prions in the ileum of lambs was detected by immunohistochemical staining for the disease-associated form of the prion protein, PrP^{Sc}. Odds ratios (OR) were determined for the frequency of PrP^{Sc} staining within enteric somata categorized by plexus location (myenteric, submucosal) and neurochemical staining (PGP 9.5, neural nitric oxide synthase, somatostatin, substance P, and vasoactive intestinal polypeptide). PrP^{Sc} was observed in $4.48 \pm 4.26\%$ of myenteric neurons and $2.57 \pm 1.82\%$ of submucosal neurons in five lambs aged 208–226 days but not in a lamb aged 138 days. The relative frequency of PrP^{Sc} within enteric somata was interdependent on plexus location and neurochemical type. Interestingly, PrP^{Sc} was observed more frequently within myenteric neurons than in submucosal neurons (PGP 9.5; OR = 1.72, 95% confidence interval = 1.21–2.44), and was observed within the myenteric plexus approximately 4× (2.16–6.94) more frequently in somatostatin neurons than in the general neural population stained by PGP 9.5. Nerve fibers stained for somatostatin were present in the mucosa and near PrP^{Sc} staining within Peyer's patches. The results suggest that somatostatin-expressing enteric neurons, with fiber projections near Peyer's patches, but with somata present in greatest proportion within the myenteric plexus, are an early target for neuroinvasion by scrapie prions and could serve an important role in neural dissemination.

Keywords Prion · Scrapie · Enteric nervous system · Somatostatin · Peyer's patches

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Introduction

Prion diseases—or transmissible spongiform encephalopathies (TSEs)—are a group of slowly progressive, fatal neurologic diseases caused by unconventional agents called prions [39]. Although some debate persists regarding the exact nature of prions, disease is critically associated with the conversion of cellular host prion protein (PrP^{c}) to TSE-associated isoforms (PrP^{TSE}) [7, 38]. Natural prion disease occurs in humans (Creutzfeldt–Jakob disease and others) and in several types of animals significant to agriculture (scrapie of sheep and goats and bovine spongiform encephalopathy of domestic cattle) and to wildlife management (chronic wasting disease of deer and elk).

Disease transmission commonly occurs via the alimentary tract but specific knowledge about how prions disseminate from the gut to the brain is incomplete (see reviews by Mabbot and MacPherson [28] and by Beekes and McBride [5]). Neuroinvasion occurs in the periphery and results in dissemination to the brain via autonomic nerves; in particular, parasympathetic and sympathetic efferent nerves projecting to the gut are thought to be common pathways.

However, the gut is most extensively innervated by a third division of the autonomic nervous system, the enteric nervous system (see reviews by Brookes and Costa [6], and Furness [15]). It is estimated in sheep that the small intestine alone contains more than 30 million myenteric and 50 million submucosal neurons, a total population that rivals that in the spinal cord [16].

The enteric nervous system is a functionally complete, ganglionated nerve network that is organized into a myenteric plexus and a submucosal plexus, out of which all layers of the gut wall are innervated. With some variation amongst species, there is a division of labor such that most neurons which control gut motility are located within the myenteric plexus, and most neurons which control mucosal blood flow and secretion are located within the submucosal plexus. Coordination is achieved through inter-plexus enteric projections, extrinsic autonomic projections, and viscerofugal projections from the enteric nervous system to prevertebral ganglia.

The enteric nervous system includes many different neuron classes, which have been classified not only by plexus location and region of the alimentary tract, but also by somato-dendritic morphology, electrophysiology, neurochemistry, fiber projection, and physiologic action on a target tissue. Major functional classes of enteric neuron include intrinsic primary afferent neurons and several types each of interneurons, smooth muscle motor neurons, secretomotor neurons, and vasomotor neurons. Though not fully characterized, even local gut immune function is modulated by the enteric nervous system, in part through peptidergic innervation of Peyer's patches [50]. The enteric nervous system is thus a complex neural system that is intimately associated with all layers of the gut, including the mucosal layer through which prions gain entry into the host, and Peyer's patches in which prion replication occurs early in disease progression.

Given that extrinsic autonomic nerves synapse with enteric neurons [15], that enteric neurons express PrP^{c} [40] and innervate all layers of the gut, and that scrapie-associated prion protein (PrP^{Sc}) is first detected in sheep within the enteric nervous system [3, 19, 46], it is hypothesized that neuroinvasion of the enteric nervous system is important for the dissemination of scrapie prions to the brain via autonomic nerves.

Many observations suggest that neurons projecting into sites of prion replication (Peyer's patches) should be targets for invasion by prions [5, 28], but a more diverse group of neurons (with projections into the mucosa or around vessels) may be exposed immediately following uptake of prions from the gut lumen [25]. The purpose of this study was to identify enteric neural targets of scrapie prions early in disease progression in a natural disease model in lambs. The results demonstrate that following neonatal alimentary disease transmission, frequency of PrP^{Sc} detection within ileal enteric neurons is disproportionately greater for myenteric neurons expressing the peptide transmitter, somatostatin. The identification of enteric neural targets of scrapie prions early in disease progression contributes significantly in understanding the factors involved in peripheral neuroinvasion and neural dissemination of prions.

Materials and methods

Oral inoculation model

All animal experiments were approved by the Washington State University Institutional Animal Care and Use Committee. The study included seven Montadale or Montadale-eXSuffolk lambs born to Montadale ewes. All lambs were VRQ/VRQ (see "Genotyping" section below), and on the day of birth were fed half a cotyledon from a placenta shed previously from an unrelated ARQ/VRQ ewe clinically affected with scrapie and with staining for PrP^{Sc} in sections of medulla at the level of the obex. Cotyledons were stored at −20°C until used.

Peripheral prion replication in lambs was assessed antemortem by third eyelid [31] or rectal [13, 18] biopsy obtained at 4–6 months of age. Lambs were humanely euthanized at about 7 months of age by intravenous injection of sodium pentobarbital (Fatal-Plus; Vortech Pharmaceuticals, Dearborn, MI). Tissues collected immediately postmortem included medulla, thoracolumbar spinal cord, celiacomesenteric ganglia, ileocecal lymph node, ileocecal junction, and a 30 cm length of ileum ending within ~3 cm of the ileocecal junction. All tissues were fixed by immersion in 10% neutral-buffered formalin; the ileum was also gently distended with formalin.

Genotyping

Approximately 100 ng DNA was used as a template for amplification of the open reading frame of PRNP using the following primer pair: 5′-GGCATTTGATGCTGACACC-3′ and 5′-TACAGGGCTGCAGGTAGAC-3′, corresponding to nucleotide positions 22234–22252 and 23106–23125 of GenBank accession no. U67922. Reactions were performed in a final volume of 90 µL under the following conditions: 1× Qiagen PCR buffer, 2.5 mM $MgCl_2$ and 2.5 U Taq polymerase (201225, TaqPCR Core kit; Qiagen, Valencia, CA). Amplification was performed under a temperature regime of 95°C for 5 min, 62°C for 30 s, and 72°C for 59 s for one cycle; 95°C for 30 s, 62°C for 30 s, and 72°C for 59 s for 30 cycles; and 95°C for 30 s, 62°C for 30 s and 72°C for 7 min for one cycle. Amplified products were

sequenced by standard dideoxynucleotide analysis (Amplicon Express, Pullman, WA) using primer pair 5′-CTGGGGTC AAGGTGGTAGCC-3′ and 5′-GGTGGTGACTGTGT-GTT GCTTGA-3′, corresponding to nucleotide positions 22553–22573 and 22838–22860 of GenBank accession no. U67922. Genotypes are reported as the deduced amino acid sequences at codons 136 (alanine, A; valine, V), 154 (arginine, R; histidine, H), and 171 (glutamine, Q; arginine, R) of the diploid genotype.

PrP^{Sc} detection by ELISA

A commercial test developed for lymphoid tissue (HerdChek Chronic Wasting Disease [CWD] Antigen Test kit; IDEXX, Westbrook, Maine) was used for detection of PrP^{Sc} in unfixed cotyledons as previously described [2]. This test is used for the postmortem detection of PrP^{Sc} in white-tailed deer retropharyngeal lymph node tissue but is also suitable for the detection of PrP^{Sc} in sheep cotyledons; the kit utilizes a PrP^{Sc} ligand immobilized on the surface of the CWD-antigen capture plate and monoclonal antibodies that recognize the ovine PrP protein. Briefly, 300 mg of cotyledon was homogenized twice in a FastPrep instrument (Qbiogene, Carlsbad, CA) for 30 s each at maximum speed (6.5 m/s) in a disruption tube containing ceramic beads and 0.9 mL of distilled, deionized water. The ELISA was then conducted according to the manufacturer's protocols. Interpretation of samples analyzed by ELISA was performed by comparing the A_{450} of the cotyledon samples with that of negative- and positive-control samples supplied with the kit (reference wavelength = 620 nm). Samples were considered positive if the mean A_{450} values were greater than or equal to the cutoff value as calculated by the test manufacturer.

Immunohistochemistry (IHC)

All formalin-fixed tissues were placed in cassettes, incubated for 60 min in 98% formic acid, washed extensively in distilled water, and re-equilibrated in formalin for 24 h, prior to paraffin embedding. Thin (3–5 μm) paraffin sections were mounted on Superfrost Plus slides, air dried overnight, and then baked at 57°C for 100 min. IHC staining was carried out using a BenchMark automated processor (Ventana Medical Systems, Tucson, AZ) with modifications to the technique previously described [31, 43]. Antigen retrieval was achieved by 60–90 min heated incubation in CC1 buffer (Ventana Medical Systems). Scrapie-associated prion protein (PrP^{Sc}) was detected using a cocktail of well-characterized monoclonal antibodies (F89/160.1.5 and F99/97.6.1; Table 1) applied for 32 min at 37°C. Evaluation of PrP^{Sc} dissemination was determined using Basic AEC Detection Kit (760–020; Ventana Medical Systems). Slides were then counterstained using Bluing Reagent and Hematoxylin kits (Ventana Medical Systems) and coverslipped in aqueous mounting media (S3025; Dako, Carpinteria, CA). In dual fluorescence labeling studies, an antibody directed toward a neurochemical of interest (Table 1) was included in the cocktail of anti-PrP^{Sc} primary antibodies. Relevant combinations of fluorescently-labeled secondary antibodies were applied for 20 min at 37°C (Table 2). Fluorescently labeled slides were coverslipped using Prolong Gold antifade reagent (P36930; Molecular Probes, Eugene, OR).

Using these techniques, PrP^{Sc} staining could be detected in the obex, lymph nodes, and ileum from a clinically affected ewe but not from an unexposed, clinically normal ewe. IHC staining for all neural and glial marker antibodies was dependent upon inclusion of the primary antibody and an appropriate fluorophore-conjugated secondary antibody.

Table 1 Primary antibodies

Target	Name	Species and isotype	Working dilution/ concentration	Source (reference)
PrP^{Sc}	F89/160.1.5	Mouse IgG_1	5 μg/mL	Dr. Katherine O'Rourke, USDA-ARS, Pullman, WA [30, 31]
	F99/97.6.1	Mouse IgG_1	5 μg/mL	Dr. Katherine O'Rourke, USDA-ARS, Pullman, WA [31]
Glial fibrillary acid protein	GFAP	Rabbit IgG	1:400	Z0334; Dako, Carpinteria, CA [24, 45]
Elav protein (human neuronal protein, Hu)	Hu	Mouse IgG_{2b}	1:200	A-21271; Molecular Probes, Eugene, OR [8]
Neural nitric oxide synthase	NOS	Rabbit IgG	1:200	SA227; Biomol Research Laboratories, Plymouth Meeting, PA [29]
Protein gene product 9.5	PGP	Rabbit IgG	1:200	Z5116; Dako
Somatostatin	SOM	Rabbit IgG	1:1,600	20067; ImmunoStar
Substance P	SP	Rat IgG_{2a}	1:50	MAB356; Chemicon, Temecula, CA
Vasoactive intestinal polypeptide	VIP	Guinea pig IgG	1:800	T-5030 (GHC7161); Peninsula Laboratories [32]

Table 2 Secondary antibodies used in fluorescence IHC

Target visualized	Antibody	Source
PrP^{Sc}, Hu	Cy3 AffiniPure donkey anti-mouse IgG	715-165-150; Jackson ImmunoResearch Laboratories, West Grove, PA
SP	Cy3 AffiniPure donkey anti-rat IgG	712-165-150; Jackson ImmunoResearch Laboratories
PrP^{Sc}	Alexa Fluor 488 goat anti-mouse IgG	A-11029; Molecular Probes, Eugene, OR
GFAP, NOS, PGP, SOM	Alexa Fluor 488 goat anti-rabbit IgG	A-11034; Molecular Probes, Eugene, OR
VIP	Alexa Fluor 488 goat anti-guinea pig IgG	A-11073; Molecular Probes, Eugene, OR

Microscopy

Slides were viewed and photographed using an Axio Imager.M1 microscope (Carl Zeiss Microimaging, Thornwood, NY) equipped with an LED illuminator for bright field microscopy and an X-Cite 120 Fl Illuminating system (EXFO Photonic Solutions, Mississauga, Ontario, Canada) for epi-fluorescence microscopy. Cy3 and Alexa Fluor 555 fluorescence were observed using the Carl Zeiss filter set 00 (excites 530–585 nm, dichroic mirror 600, 615 nm long-pass filter), and Alexa Fluor 488 fluorescence was observed using the Carl Zeiss filter set 38HE (excites 450–490 nm, dichroic mirror 495, 500–550 nm band-pass filter). The microscope was equipped with an AxioCam MRc5 digital camera (Carl Zeiss Microimaging) connected to a computer workstation running AxioVision 4.5 imaging software (Carl Zeiss Microimaging). For presentation in Figs. 4 and 7, maximum intensity projections were produced from sequentially scanned z-stack series (0.5–1 μm intervals) using an LSM 510 META laser scanning microscope (Carl Ziess Microimaging) equipped with 488 and 543 nm lasers (pinholes set to 1 Airy unit).

Statistical methods

All analyses were performed using SAS for Windows (ver. 9.1.3, service pack 4; SAS Institute, Cary, NC). The data set was categorized by animal, enteric plexus location (myenteric or submucosal) and by neurochemical type (PGP, nNOS, SOM, SP, or VIP; abbreviations defined in Table 1). The total number of neurons (defined by somata staining) and the number of those colocalized with PrP^{Sc} were recorded for each neurochemical type. The total association of PrP^{Sc} colocalization in neurochemically-defined (NOS^+, SOM^+, SP^+, or VIP^+) somata compared to colocalization in the total neural population (PGP^+) was determined for each animal by Fisher's exact testing. The null hypothesis of random detection of PrP^{Sc} amongst neurochemically-defined enteric neurons was assessed by conditional logistic regression using a generalized linear mixed model, the GLIMMIX procedure (rel. June 2006; SAS Institute), and the fixed effects of plexus location, neurochemical type, and the interaction of these two effects. The regression was conditioned by including the random effect of subjects (lambs) and adjusted for overdispersion by including the random effect of residuals. Holm-adjusted P values (P_{adj}) were produced using the method of Bonferroni in logical step-down fashion [23]. Odds ratios (ORs) and corresponding upper and lower CL_{adj}s are the exponentiation of the least squares means parameter and CL_{adj}s estimates, respectively. Least squares means parameter and Holm-adjusted 95% confidence limit (CL_{adj}) estimates were produced for two "families" of post-hoc comparisons of interest; family-wise error rate, $\alpha = 0.05$.

Results

Alimentary transmission and staging disease progression

Cotyledons randomly selected from the pool of cotyledons used for alimentary inoculation had PrP^{Sc} staining by IHC and a PrP^{Sc} ELISA titer between 1:64 and 1:256. Ewes giving birth to the lambs were not a likely source of PrP^{Sc} since PrP^{Sc} staining was neither detected by IHC in antemortem peripheral lymphoid tissues (third eyelid and rectal mucosa) and postmortem obex, nor by IHC and western blot in shed cotyledons randomly sampled at the birth of these lambs.

Tissues from a lamb euthanized before 2 weeks of age due to an unrelated illness were not collected. In the remaining six lambs, PrP^{Sc} was detected antemortem in rectal mucosa-associated lymphoid tissue between 124–219 days of age, and in ileal Peyer's patches and ileocecal lymph node collected postmortem between 138–226 days of age (Fig. 1b, d, f; scrapie-negative control tissues shown in Fig. 1a, c, e). PrP^{Sc} was not detected in the central nervous system (medulla and intermediolateral column of T8–L3 spinal cord segments) or celiacomesenteric ganglia (138–226 days of age), except in one lamb (226 days of age) in which sparse punctate staining in celiacomesenteric ganglia appeared to be located mostly in satellite cells (Fig. 2a–d). PrP^{Sc} was detected in the enteric nervous system of the ileum in five lambs aged 208–226 days, but not in a lamb aged 138 days (examples shown in Fig. 3). The appearance of PrP^{Sc} staining within enteric somata was generally punctate (Fig. 3d–f, Fig. 4a–c) but sometimes

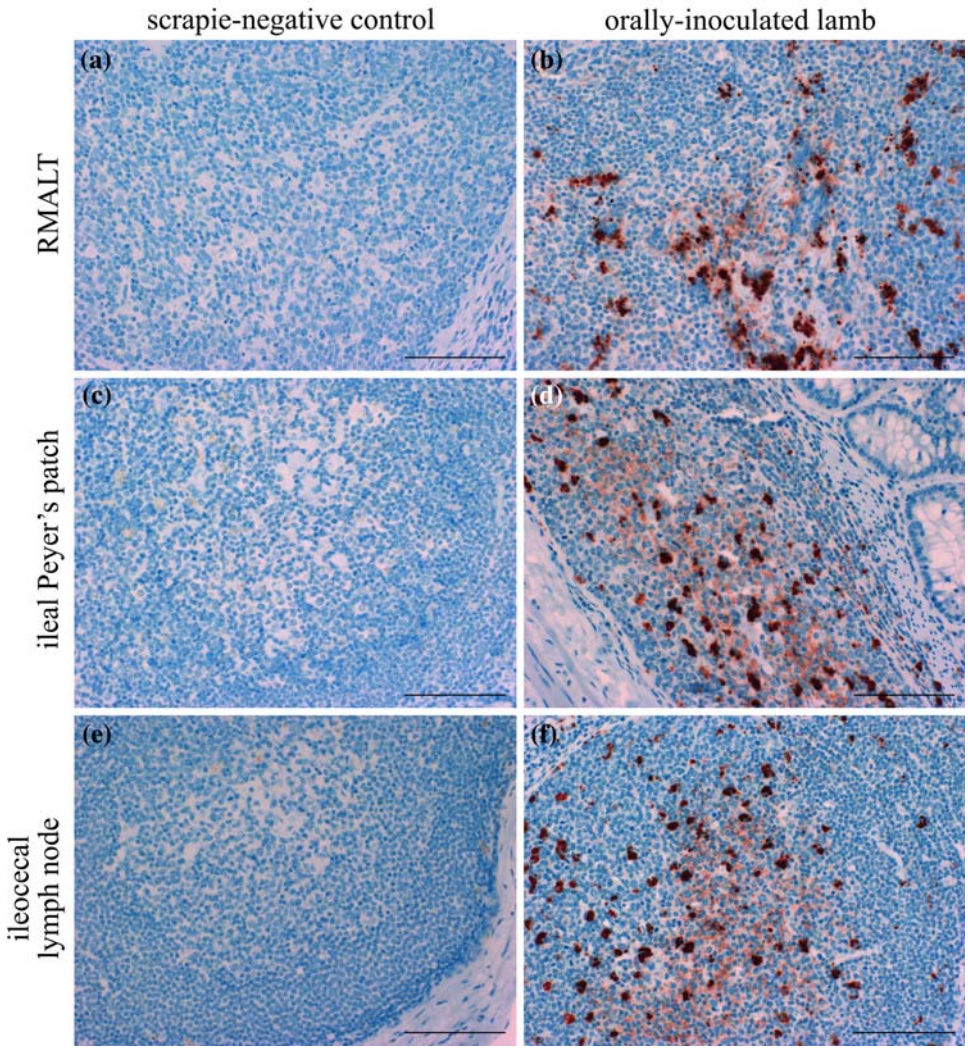

Fig. 1 PrP^{Sc} staining (*red*) was not observed in various lymphoid tissues from a scrapie-negative control ewe (**a**, **c**, **e**) but was readily detected in lymphoid tissues from a lamb orally inoculated with scrapie prions at birth (**b**, **d**, **f**). Lymphoid tissues shown are from: **a**, **b** rectal mucosa-associated lymphoid tissue (*RMALT*); **c**, **d** *ileal Peyer's patch*; **e**, **f** *ileocecal lymph node*. *Bars* 100 μm

globular when near cell margins. PrP^{Sc} was also occasionally observed within enteric glia (Fig. 4d–f).

Frequency of PrP^{Sc} detection within defined populations of enteric neurons

The occurrence of PrP^{Sc} in PGP^+ somata was tallied for a total of 10,841 myenteric neurons and 19,495 submucosal neurons. The frequency of PrP^{Sc} detection in PGP^+ somata ranged from 1.21 to 11.67% (mean and standard deviation: $4.48 \pm 4.26\%$) in myenteric neurons, and from 1.01 to 5.68% ($2.57 \pm 1.82\%$) in submucosal neurons ($n = 5$ lambs). Frequencies of PrP^{Sc} detection in enteric neurons defined by plexus location and neurochemical type are shown for each lamb in Fig. 5. The pattern of PrP^{Sc} detection frequency amongst eight enteric neural subgroups relative to that in PGP^+ somata varied between lambs except for a consistently increased detection frequency in SOM^+ neurons of the myenteric plexus (Breslow–Day test for homogeneity: Chi-square = 1.7067, $P = 0.7895$).

The significance and strength of the association of PrP^{Sc} detection frequency with subpopulations of enteric neurons was measured by odds ratio estimates for somata defined by plexus location and neurochemical type and analyzed by conditional logistic regression. The odds of PrP^{Sc} detection were significantly dependent upon plexus location ($F = 4.66$, $P = 0.0376$), neurochemical type ($F = 20.59$, $P < 0.0001$), and the interaction of these main effects ($F = 6.66$, $P = 0.0004$). The detailed post-hoc analyses of this finding are summarized below and in Fig. 6.

PrP^{Sc} was detected more frequently in the myenteric plexus versus the submucosal plexus (Fig. 6a) for the general population of neurons (PGP; 1.72, 1.21–2.44) and for the subpopulation of SOM^+ neurons (3.92, 2.09–7.35). Relative to local PGP^+ neural populations, the frequency of PrP^{Sc} detection was significantly increased for only SOM^+ neurons in the myenteric plexus (3.87, 2.16–6.94; Fig. 6b) and in the submucosal plexus (1.70, 1.02–2.82; Fig. 6c). Significantly lower frequencies of detection were associated with SP^+ neurons in the myenteric plexus (0.21, 0.05–0.87;

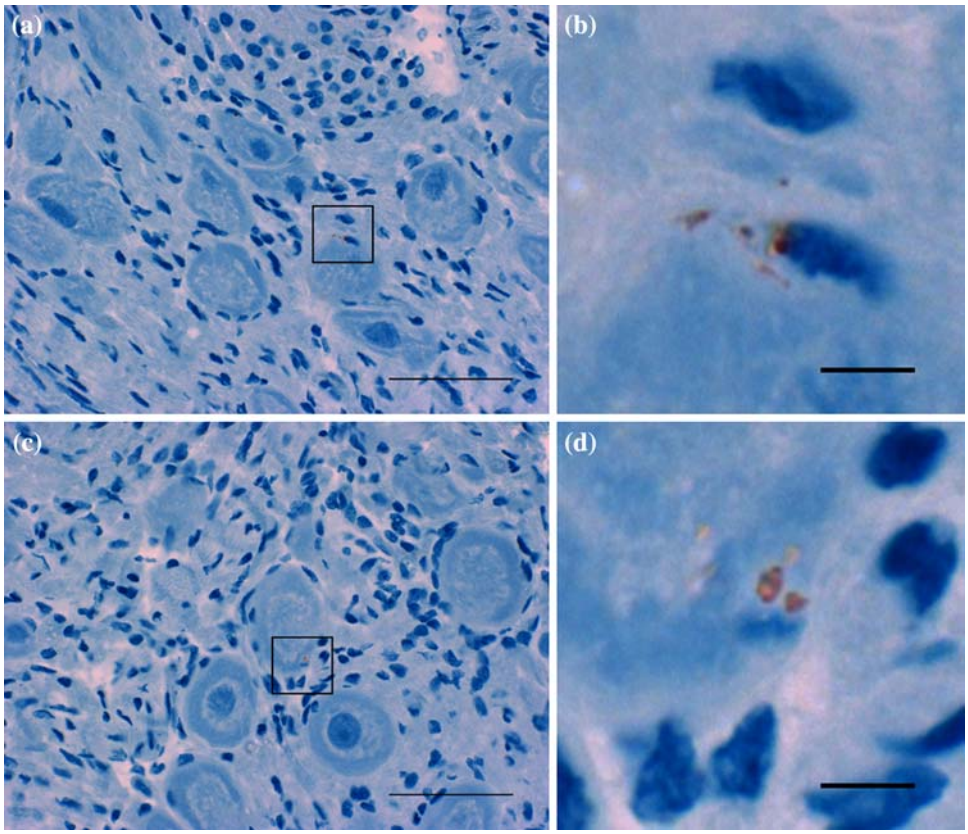

Fig. 2 Detection of PrP^{Sc} staining (*red*) in the celiacomesenteric ganglia from a lamb aged 226 days following oral inoculation with scrapie prions at birth. **a** PrP^{Sc} detected in a satellite cell. The *box* depicts the region shown at higher magnification in **b**. **c** PrP^{Sc} at the margin of this ganglion cell may also be located in a satellite cell in which only a part of the nucleus is present in this section. The *box* depicts the region shown at higher magnification in **d**. *Bars* 50 μm (**a**, **c**), 5 μm (**b**, **d**)

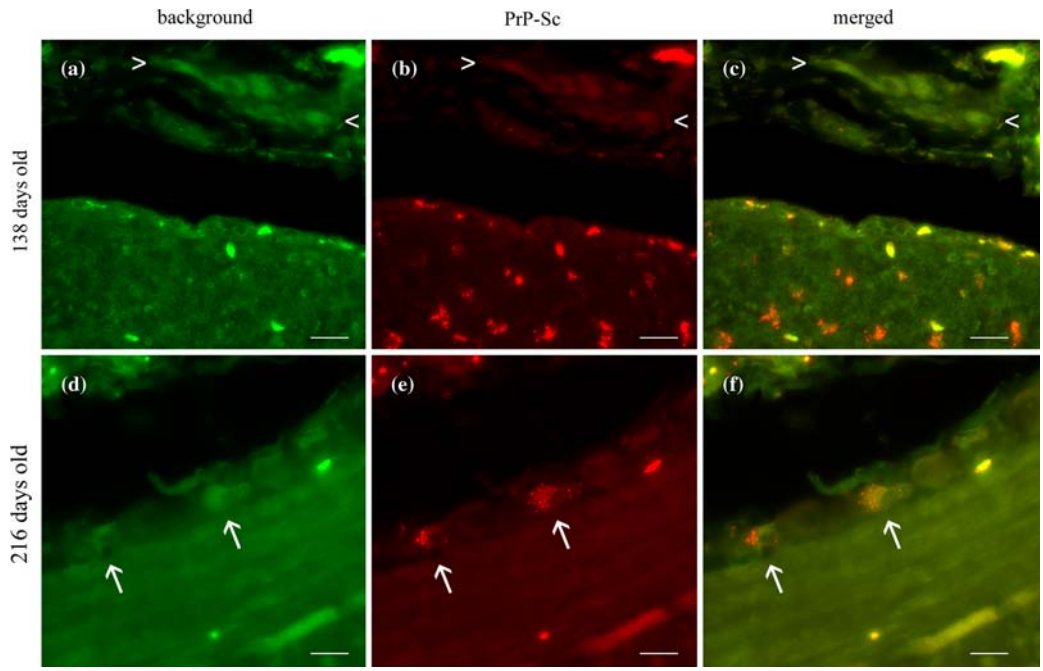

Fig. 3 Examples of PrP^{Sc} staining (*red*) detected in the ileum of two lambs orally inoculated with scrapie prions at birth. **a–c** In a lamb aged 138 days, PrP^{Sc} staining is present within a Peyer's patch follicle (*lower half* of image) but not within adjacent submucosal neurons (*arrowheads* denote ganglion limits). **d–f** PrP^{Sc} staining in two submucosal neurons (*arrows*) of a lamb aged 216 days. PrP^{Sc} staining was also present in a Peyer's patch follicle located out of frame *above* this ganglion. The *background* (*green*) column is included to provide tissue contrast. PrP^{Sc} staining remains *red* in the *merged* column of this single labeling experiment, whereas autofluorescence appears *bright yellow*. Images are maximum intensity projections from 6 μm z-stacks. *Bars* 20 μm

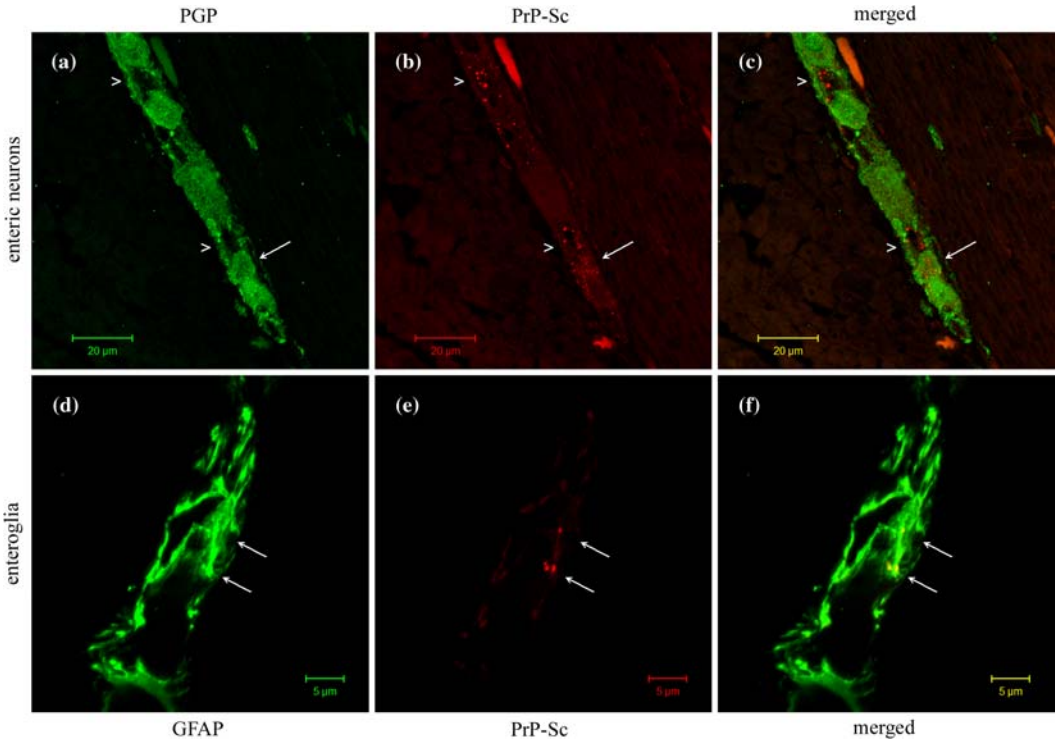

Fig. 4 Initial detection of PrP^{Sc} staining (*red*) in the enteric nervous system of the ileum from a lamb aged 216 days following oral inoculation with scrapie prions at birth. **a–c** PrP^{Sc} detected within (*arrow*), and outside of (*arrowheads*), PGP^{+} enteric neurons (*green*). **d–f** PrP^{Sc} detected within (*arrows*) $GFAP^{+}$ enteric glia (*green*). Images are maximum intensity projections from 2.5 μm (**a–c**) and 1.5 μm (**d–f**) confocal z-stacks. *Abbreviations* defined in Table 1

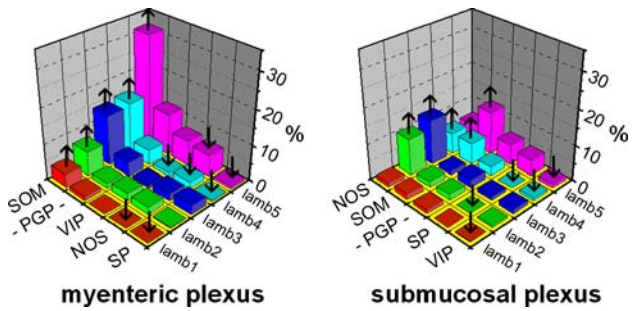

Fig. 5 Frequency of detecting PrP^{Sc} staining within subpopulations of enteric neuron in the ileum of lambs early in disease progression following oral inoculation with scrapie prions at birth. Note the consistent increase in the frequency of PrP^{Sc} detection within myenteric plexus SOM^{+} neurons as compared to that in the general myenteric neuron population (*-PGP-*). Each *bar color* represents data from a lamb (lambs 1–5) at approximately 7 months of age. Frequency of PrP^{Sc} detection is given as the percent (*%*, *vertical axis*) within each type of neuron per lamb. *Abbreviations* are defined in Table 1. *Arrows* indicate the direction of significant differences (Fischer's exact test; $P < 0.05$)

Fig. 6b) and VIP^{+} neurons in the submucosal plexus (0.23, 0.07–0.76; Fig. 6c).

SOM^{+} nerve fibers in the ileum

SOM^{+} nerve fibers were frequently observed projecting between mucosal epithelium and Peyer's patches with PrP^{Sc} staining (Fig. 7). Similar to a previous description [49], staining for SOM was also occasionally observed in "open-type" epithelial cells of the ileal mucosa (not shown).

Discussion

The process of neuroinvasion of the enteric nervous system by prions was studied in the ileum of sheep, the first peripheral region in which neuroinvasion occurs in naturally acquired disease [3, 12, 47]. We determined that the relative frequency of PrP^{Sc} staining, used as a surrogate marker [1, 26] for scrapie prions within enteric neurons, is dependent upon that neuron's plexus location and neurochemical type. In particular, SOM^{+} myenteric neurons were identified as a consistent target for neuroinvasion early in disease progression. From the evidence, we postulate that a significant subgroup of SOM^{+} neurons innervate compartments of Peyer's patches where efficient neuroinvasion is thought to occur, and represent an efficient pathway for neural dissemination within the small intestine. We expect that prion invasion of neurons that do not convert PrP^{c} into PrP^{Sc} that is readily detectable by immunohistochemistry [4], should they exist within the enteric nervous system, would be underrepresented in the present study.

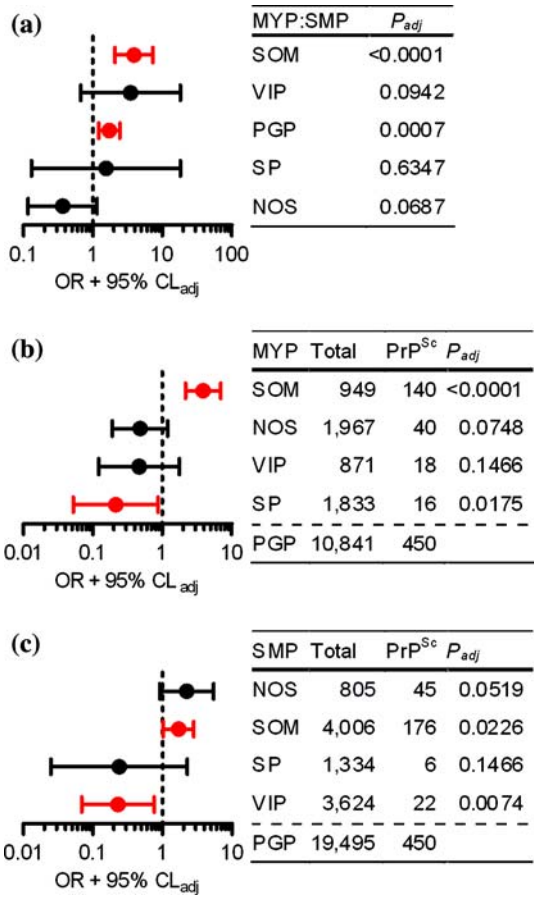

Fig. 6 The relative frequency of detecting PrP^{Sc} staining within ileal enteric neurons of lambs is interdependent on plexus location and neurochemical type early in disease progression. **a** The frequency of PrP^{Sc} detection was greater in the myenteric versus submucosal plexus for the general (*PGP*) neural population and for the subpopulation of SOM^+ neurons. **b**, **c** SOM^+ neurons were the only subpopulation studied that had an increased frequency of PrP^{Sc} detection in either plexus (**b** myenteric, **c** submucosal), relative to that in the respective general neural population. The difference between an odds ratio (OR) and 1 (*dotted vertical lines*) indicates the relative strength of the association with the frequency of PrP^{Sc} detection. ORs significantly greater or less than 1 (depicted in *red*; $P_{adj} < 0.05$) indicate associations with increased or decreased frequency, respectively, of PrP^{Sc} detection. CL_{adj} Holm-adjusted confidence limits, P_{adj} Holm-adjusted *P* values, PrP^{Sc} the number of neurons in which PrP^{Sc} was detected, *MYP* myenteric plexus, *SMP* submucosal plexus, *Total* the total number of neurons counted. See Table 1 for abbreviations of neurochemical types. *y-axis* First column, *x-axis* $\log_{10}$

SOM^+ enteric neurons are targets of peripheral neuroinvasion by scrapie prions

There is consensus that transport of prions across gut epithelium and exposure of mucosal nerve fibers are processes relevant to peripheral neuroinvasion and neural dissemination [5, 26, 28, 36, 47]. Considering that the greatest proportion of mucosa-projecting neurons typically occurs in the submucosal plexus of large mammalian species [44], it is surprising that PrP^{Sc} detection early in disease was most frequent in neurons located in the myenteric plexus (Fig. 6a, PGP), and was relatively infrequent in a major type of submucosal neuron (Fig. 6c, VIP). Further, PrP^{Sc} detection in SOM^+ neurons of the myenteric plexus was very consistent and occurred at nearly 4× greater frequency (Fig. 6b).

SOM^+ enteric neurons of the small intestine generally belong to one of two classes that differ in function and projection: secretomotor neurons that project to the mucosal layer, and descending interneurons that synapse with other enteric neurons [6]. Determining the projections of SOM^+ enteric nerve fibers in sheep is thus particularly germane to locating a compartment in which efficient neuroinvasion occurs. Given the present data, it is conceivable that SOM^+ neurons targeted by scrapie prions are distributed in greatest proportion within myenteric ganglia but which, nevertheless, project to mucosal compartments in which efficient neuroinvasion occurs early in disease progression.

Implications of SOM^+ secretomotor neurons targeted by prions

Jeffrey et al. [25] observed, soon after the injection of infectious material into the lumen of the small intestine of lambs, that PrP^{Sc} staining was widespread within mucosal villi. Mucosal villi are highly innervated by enteric neurons, including fibers of secretomotor neurons, which travel close to the epithelium to affect its function [21, 22, 41]. Fibers of numerous mucosa-projecting neurons, especially secretomotor neurons, are thus potentially exposed upon mucosal transportation of prion and prior to replication of prion within Peyer's patches.

In sheep, mucosal nerve fibers include those that express VIP, SP, NOS [8, 27, 51], and also SOM (Fig. 7). Furthermore, the secretomotor action of VIP, SP, and SOM is typically preserved within large mammalian species [44]. Therefore, it is not surprising that PrP^{Sc} was detected in each of these neurochemical types of submucosal neuron early in disease progression following alimentary transmission in sheep. That PrP^{Sc} was detected as much as 4× more frequently in SOM^+ neurons, however, may indicate that efficient enteric neuroinvasion occurs in a mucosal compartment uniquely innervated by SOM^+ neurons. Evidence implicates such a compartment is associated with Peyer's patches.

Neuroinvasion occurring via nerve fiber projections to Peyer's patches is consistent with the typical detection of PrP^{Sc} first within Peyer's patches and then nearby ganglia, and with close associations between fibers and PrP^{Sc}-laden cells [3, 20]. Recent evidence in mice further demonstrates that efficient neuroinvasion following alimentary transmission is dependent upon follicular dendritic cells [17], a cell type

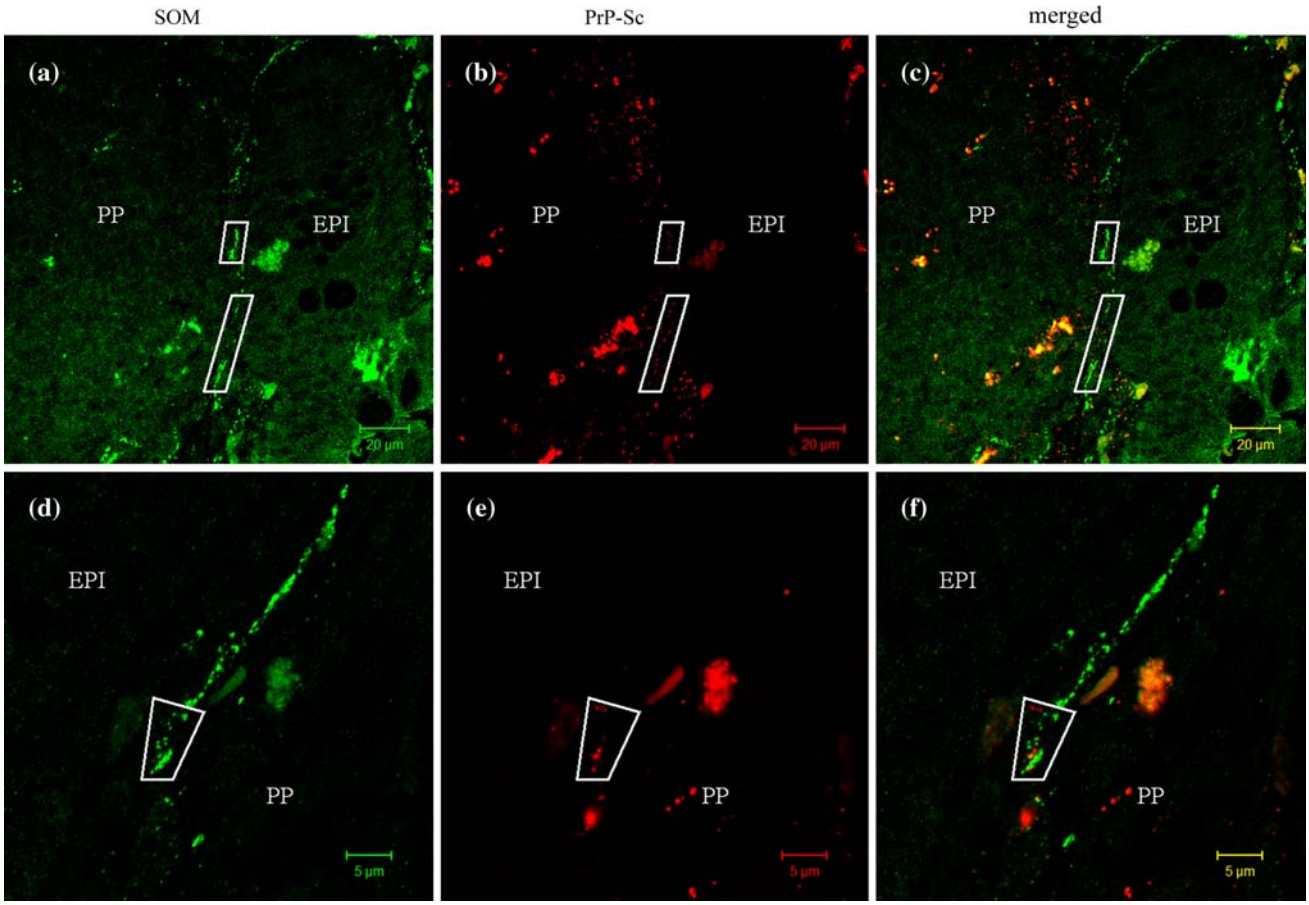

Fig. 7 Nerve fibers stained for SOM in the mucosal layer of the ileum from a lamb aged 208 days and orally inoculated with scrapie prions at birth. **a**–**c**, **d**–**f** Two examples of SOM^+ (*green*) nerve fibers projecting between the mucosal epithelium (*EPI*) and a Peyer's patch (*PP*) with PrP^{Sc}-staining (*red*). Note the relatively close association of SOM^+ fibers with PrP^{Sc} staining (*boxes*). Images are maximum intensity projections from 6 µm (**a**–**c**) and 2 µm (**d**–**f**) confocal z-stacks. *SOM* somatostatin

associated with nerve fibers in the interfollicular zone and dome of Peyer's patches [11]. Although SOM^+ Peyer's patch fibers are yet to be confirmed in mice [10, 48], these have been observed in cats [14], and now also in sheep (Fig. 7). It is thus conceivable that in sheep, efficient neuroinvasion near Peyer's patches is dependent upon proximity of SOM^+ nerve fibers to PrP^{Sc}-laden immune cells or exosomes [35, 37].

Implications of SOM^+ descending interneurons targeted by prions

A subgroup of descending myenteric interneurons of the small intestine expresses SOM [6]. In the present study in sheep, prion targeting of SOM^+ descending interneurons would imply neuroinvasion early in disease occurred within ganglia, in contrast to that occurring outside of ganglia via nerve fibers. Nerve endings of extrinsic nerves are unlikely to have been a significant source of prion within enteric ganglia since PrP^{Sc} was first detected in enteric neurons. Prions might alternatively enter ganglia from a non-neural source, such as blood. However, if blood-borne prion were relevant to enteric neuroinvasion early in disease, then it would be reasonable to expect relatively higher frequency PrP^{Sc} detection in vasomotor neurons.

Vasomotor neurons have not been extensively studied in large mammalian species but probably include subgroups of VIP^+ and SP^+ neurons [44, 51]. The relative frequency of PrP^{Sc} detection in VIP^+ and SP^+ neurons, however, was not increased in either plexus (Fig. 6b, c). Therefore, simple prion entry from a non-neural source such as blood does not likely account for the higher frequency of PrP^{Sc} detection within SOM^+ neurons. Regardless, detection of PrP^{Sc} in SOM^+ descending interneurons early in disease progression has implications for neural dissemination.

SOM^+ descending interneurons form a synaptic network that may descend the length of the small intestine—from duodenum to ileum, at least in guinea pigs [9, 33, 34, 42]. If this also occurs in sheep, then prion dissemination through SOM^+ descending interneurons might account for the observed oral spread of prion from the ileum to duodenum [3, 47].

In summary, following neonatal alimentary transmission in sheep, immunohistochemical detection of PrP^{Sc} (as a surrogate marker for neuroinvasion by scrapie prions) in ileal enteric neurons was interdependent on the neuron's plexus location and neurochemical type. Specifically, SOM^+ neurons, especially those located in the myenteric plexus, were found to be targets of neuroinvasion early in disease progression. These results are consistent with the hypothesis that efficient neuroinvasion occurs near Peyer's patches in compartments innervated by SOM^+ neurons. In addition, neuroinvasion of SOM^+ enteric neurons early in disease progression could explain the neural dissemination observed within the small intestine of sheep with naturally acquired disease.

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