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REGULAR ARTICLE

Neuroimmune connections in ovine pharyngeal tonsil: potential site for prion neuroinvasion

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Abstract Recent studies have established the involvement of nasal-associated lymphoid tissues, mainly the pharyngeal tonsil, in prion pathogenesis. However, the mechanisms of the associated neuroinvasion are still debated. To determine potential sites for prion neuroinvasion inside the ovine pharyngeal tonsil, the topography of heavy (200 kDa) and light (70 kDa) neurofilaments and of glial fibrillar acidic protein has been semi-quantitatively analysed inside the various compartments of the tonsil. The results show that the most innervated areas are the interfollicular area and the connective tissue located beneath the respiratory epithelium. The existence of rare synapses between follicular dendritic cells and nerve fibres inside the germinal centre indicates that this mechanism of neuroinvasion is possible but, since germinal centres of lymphoid follicles are poorly innervated, other routes of neuroinvasion are likely. The host *PRNP* genotype does not influence the pattern of innervation in these various tonsil compartments, unlike ageing during which an increase of nerve endings occurs in a zone of high trafficking cells beneath the respiratory epithelium. A minimal age-related increase of innervation inside the lymphoid follicles has also been observed. An increase in nerve fibre density around the lymphoid follicles, in an area rich in mobile cells such as macrophages and dendritic cells capable of capturing and conveying pathogen prion protein (PrPd), might ensure more efficient infectivity, not in the early phase but in the advanced phase of lymphoinvasion after the amplification of PrPd; alternatively, this area might even act as a direct site of entry during neuroinvasion.

Keywords Sheep · Scrapie · Pharyngeal tonsil · Follicular dendritic cell · Innervation

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Introduction

Scrapie, an endemic disease of sheep and goats, belongs to the transmissible spongiform encephalopathies (TSEs), a family of neurodegenerative diseases affecting humans and animals. The TSEs also include Creutzfeld-Jakob disease (CJD) and its

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variant (vCJD) in humans, bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease (CWD) in mule deer. They are caused by the aggregation of aberrantly folded "prion protein", initially described by Griffith (1967) and refined by Prusiner (1982). This pathogen protein, PrPd, is an isoform of a host-encoded protein, PrPc, physiologically expressed by most cells such as nerve, lymphoid and muscular cells (Horiuchi et al. 1995). Expression of this normal cell protein is essential to TSE pathogenesis. In contact with PrPd, PrPc undergoes a conformational change leading to pathogenic conversion (Weissmann 2004).

In sheep, the genotype of the host modulates susceptibility/resistance to scrapie, which is dependent on various polymorphisms throughout the PrP gene (*PRNP*). Mutations at positions 136, 154 and 171 of the PrP chain strongly influence the susceptibility to scrapie. Among the possible genotypes derivable from this combination, the ARR/ARR genotype is strongly associated with resistance to scrapie, whereas VRQ/VRQ is clearly associated with susceptibility (Baylis et al. 2004). Depending on the breed of the sheep, the ARQ/ARQ genotype can be associated with resistance or with susceptibility, as in Suffolk (Hunter et al. 1997) and Texel (Baylis et al. 2002) breeds.

Dissemination of prions into the environment can occur from several biological materials, e.g. infectious placentas (Race et al. 1998), saliva (Vascellari et al. 2007), urine (Seeger et al. 2005) and excreta (Miller et al. 2004). Although the alimentary tract has been suggested as the most likely port of entry in natural scrapie, several other experimentally potent routes of infectivity have been identified (Bessen et al. 2009). In animals, the olfactory system is important in social behaviour, in the search for food and in the exploration of the environment. Recent studies have focused on the role of the nasal cavity not only as a source of infection through the excretion of PrPd in nasal secretions (Bessen et al. 2010) but also as an efficient route for prion uptake and systemic dissemination. Intranasal inoculation of a scrapie strain in hamsters leads to the deposition of the pathological prion protein in the nasal associated lymphoid tissue (NALT) several weeks before detection in the vagus or trigeminal nerve nucleus in the central nervous system (CNS; Sbriccoli et al. 2009). The mechanism of neuroinvasion by prions from the NALT remains unclear. Two pathways are suspected: (1) lymphatic or haematogenous routes (Hunter et al. 2002; Halliday et al. 2005) and (2) "retrograde neuroinvasion" sustained by contact that is initiated during the preclinical phase and that occurs between PrPd-replicating cells in the lymphoreticular system (LRS) and nerve endings, followed by retrograde transport along efferent nerve fibres allowing propagation to the CNS (Van Keulen et al. 2002). In this second mechanism, the innervation of the NALT appears to be a rate-limiting step in the pathway of neuroinvasion after intranasal infection.

The pharyngeal tonsil, located in the caudal part of the pharyngeal septum, contains the largest volume of NALT and the largest epithelial surface of the nasopharyngeal parts of Waldeyer's ring (Casteleyn et al. 2007). Ultramicroscopic examination of the epithelium of the pharyngeal tonsil has revealed the presence of M cells implicated in the uptake and sampling of foreign antigens and microorganisms (Casteleyn et al. 2010; Kraehenbuhl and Neutra 2000). According to these anatomical and histological characteristics, one is tempted to speculate that the pharyngeal tonsil is an important reservoir for prion pathogen.

In this work, we have evaluated semi-quantitatively the topography of the peripheral nervous system in the lymphoid and non-lymphoid compartments of pharyngeal tonsils. For this analysis, the following variables have been taken into account: the genotype and the age of the sheep. We have highlighted neuroimmune connections between follicular dendritic cells (FDCs), strongly implicated in PrPd amplification (Jeffrey et al. 2000; Mabbott et al. 2000) and nerve fibres in the lymph follicles of pharyngeal tonsils. This observation supports their possible involvement in the neuroinvasion process and sustains the hypothesis of "retrograde neuroinvasion" from the NALT.

Materials and methods

Animals

Scrapie-free sheep (n=17) were classified into four groups according to their genotype and age: (1) French Texel lambs, 4–6 months old, of the ARR/ARR genotype (n=6), (2) French Texel lambs, 4–6 months old, of the ARR/VRQ genotype (n=4); (3) old sheep, 24–30 months old, comprising a resistant French Texel of the ARR/ARH genotype (n=1) and resistant Suffolks of the ARR/ARR genotype (n=3); (4) old susceptible French Texels of the ARQ/ARQ genotype (n=1) and ARR/VRQ genotype (n=1) and a Suffolk of the ARQ/ARQ genotype (n=1).

Sample collection

Ovines were killed at a local abattoir and pharyngeal tonsils were removed from the nasopharynx. Sections of the medial part of each tonsil were immersed in Tissue-Tek OCT embedding medium (SAKURA, Zouterwoude, The Netherlands), snap-frozen and stored at –20°C.

Cryosections

Serial transverse sections (10 μm thick) of the medial part of the tonsil were cut at –15°C with a microtome (MICRON HM 500 OM) and mounted on glass slides coated with poly-

L-lysine (Sigma, St Louis, Mo., USA), air-dried, fixed in acetone for 10 min at 4°C and then stored at –80°C until use.

Antibodies

The FDCs were stained with a mouse anti-bovine monoclonal antibody (undiluted hybridoma supernatant), FDC-B1, kindly provided by F. Melot (Melot et al. 2004).

Nerve fibres were labelled with antibodies directed against:

- Intermediate neurofilaments heavy (NFH): rabbit anti-bovine NFH 200 kDa (1/1000; AbD Serotec, Dusseldorf, Germany);
- Intermediate neurofilaments light (NFL): rabbit anti-human NFL 70 kDa (1/1000; AbD Serotec);
- Schwann cells: rabbit anti-cow glial fibrillar acidic protein (GFAP; 1/1000; Dako, Glostrup, Denmark).

NF or neuron-specific intermediate filaments are major cytoskeletal elements in neurons (Gotow 2000). They are the most abundant structural components in myelinated axons, whereas they are sparse in the perikarya and dendrites (Lee and Cleveland 1996). NF in axons are essential for conduction velocity.

GFAP is a monomeric intermediate filament protein expressed in the cytoskeleton of mature astrocytes and outside the CNS in mature non-myelin-forming Schwann cells (Notturno et al. 2008). These proteins are not found, or only at a much lower level, in myelin-forming Schwann cells (Jessen et al. 1990). In order to test the specificity of the monoclonal antibodies used, the samples were incubated with irrelevant antibodies. Negative controls were obtained by incubating samples with secondary antibodies only.

Immunoperoxidase staining

Cryosections were rehydrated and incubated for 1 h at room temperature with primary antibodies. A secondary conjugated species-specific immunoglobulin peroxidase-labelled polymer (Amplification EnVision System-horseradish peroxidase [HRP]; Dako, Glostrup, Denmark) was applied on cryosections for 30 min at room temperature. Peroxidase activity was revealed with 9-ethyl-3-aminocarbazole (Zymed, San Francisco, Calif., USA) combined with H_2O_2 as substrate.

Immunofluorescent staining

Double-immunofluorescent labelling was performed to detect simultaneously FDCs and nerve fibres. Each incubation period with the selected antibodies was performed at room temperature for 1 h. Incubations with secondary antibodies were carried out in the dark. After rehydration, sections were first incubated with FDC-B1 and then rinsed in phosphate-buffered saline. Primary antibodies were revealed with a goat anti-mouse antibody conjugated to Alexa 594 (Molecular Probes, Leiden, The Netherlands) and diluted at 1/4000. Anti-GFAP or anti-NFH antibodies were then applied and revealed with an Alexa-488-conjugated goat anti-rabbit antibody (Molecular Probes) diluted at 1/4000 and 1/8000, respectively.

Confocal analysis

All samples were observed with a Leica (Germany) SP2 confocal microscope. Cryosections of 10 μm were scanned at their best fluorescent zone, which was divided into 15 sections. Each section was analysed. Virtual colours were attributed for detection channels: red for FDCs and green for GFAP-positive (GFAP+) or NFH+ nerve fibres. The overlap of these virtual colours appeared in yellow and drew our attention to possible contact between the labelled structures. Spectral analysis of the colour channel allowed us to confirm areas of contact.

Semi-quantitative morphometrical analysis

A semi-quantitative analysis of the distribution of the immunoperoxidase-labelled nerve fibres was assessed throughout the tissue section in the following pharyngeal tonsil compartments: surface epithelium, lamina propria, interfollicular area, mantle zone (MZ) and germinal centre (GC) of the follicles and loose connective tissue constituting the central axis of the tonsil. The presence of labelled nerve fibres in each zone was scored from 0 to 5 on the basis of the following criteria: 0=absence of labelled fibre (LF); <1=less than 1 LF; 1=from 1 to 5 LF; 2=from 6 to 15 LF; 3=from 16 to 30 LF; 4=from 31 to 50 LF; 5=more than 51 LF. Numbers of follicles within each section were counted. In order to standardise our scoring method, we analysed the various compartments in our sections in total and introduced a correction in the statistical analyses. For each section, a statistical co-variable was created that took into account the surface and perimeter of (1) the lymphoid compartments and (2) the connective tissue filling the central axis, respectively and (3) the number of follicles in each tissue section. The correction of the data by using such co-variables avoided the variations in the counting linked, for example, to the higher surface area of lymphoid tissue. Six slides per tonsil were randomly selected among the serial transversal sections cut in the middle part of each tonsil. The tissue sections were immunolabelled and analysed blind to the age and the genotype of the sheep.

Statistical analyses

Statistical tests were performed by using the Statistical Analysis System (SAS 9.1.3, service pack 4). The data were

analysed by a logistic procedure measuring the influence of the independent variables (age and genotype) on dependent variables (GFAP+, NFH+ and NFL+ nerves fibres). The effect was significant if the probability was lower than 0.05.

Results

Postulating that (1) the upper respiratory tract and most likely the pharyngeal tonsil serve as a natural site of entry for scrapie agent, (2) FDCs, resident cells of the GC, are implicated in the retention and replication of scrapie agent and (3) nerve fibres probably act as the initial site of neuro-invasion for prions, we examined the innervation pattern of the ovine pharyngeal tonsil and focused our attention on the neuro-FDC interface.

Topography and semi-quantitative analysis of nerve fibres in pharyngeal tonsil

The ovine pharyngeal tonsil located in the caudal part of the pharyngeal septum was observed to be unique, had an approximate size of 3 cm in length and 1.5 cm in width and was lined on its external surface by many longitudinal exophytic folds.

Pharyngeal tonsils could be histologically separated into two areas: lymphoid and non-lymphoid compartments (Fig. 1). The non-lymphoid compartments consisted into a central axis, which was filled by loose connective tissue rich in adipose cells and that contained numerous blood and lymphatic vessels; and the deeply folded pseudostratified ciliary epithelium covering the luminal surface of the tonsil. The lymphoid compartments consisted into the lymphoid follicles composed of a GC, with resident FDCs in the light zone, clearly identified in Fig. 1 by immunostaining with FDC-B1 antibody and an MZ of naive B cells facing the epithelium; the interfollicular area lying around the lymphoid follicles; and the connective tissue, which lay under the respiratory epithelium and that was highly infiltrated by immune cells. In some locations, the epithelium was invaded by lymphoid cells, thereby losing its typical structure and providing a route to the reticular lymphoepithelium.

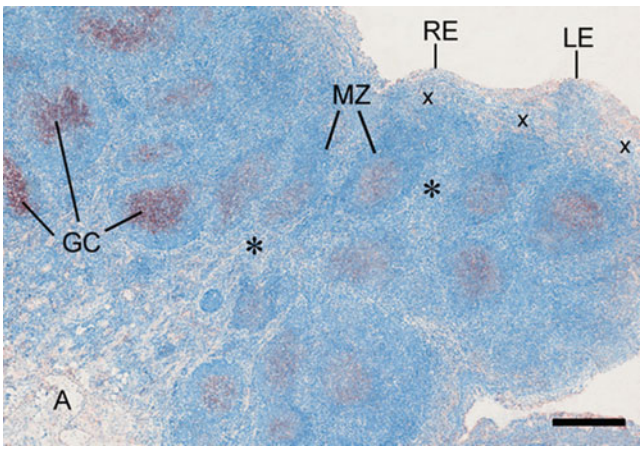

Fig. 1 Lymphoid and non-lymphoid compartments of the ovine pharyngeal tonsil. Cryosection immunolabelled with monoclonal antibody (moAb) FDC-B1. Counterstaining with haematoxylin. The pattern of innervation inside the tonsils was evaluated by semi-quantitative scoring in the various compartments (*A* central axis of the tonsil, *asterisks* interfollicular area, *GC* germinal centre of the lymphoid follicle containing the follicular dendritic cells labelled in *red*, *MZ* mantle zone, *RE* respiratory epithelium, *Le* lymphoepithelial tissue, *X* sub-epithelial connective tissue). *Bar* 500 μm

The mean scoring of the innervation in these various compartments of the pharyngeal tonsil is presented in Table 1.

In the central axis of the tonsil, many thick bundles of GFAP+ and NFH+ nerve fibres and isolated labelled fibres

Table 1 Mean scoring of innervation in the various compartments of the pharyngeal tonsil in sheep of known age and genotype (*RES* resistant, *SUSC* susceptible). The nervous fibres were abundant in the loose collagenous tissue constituting the central axis of the tonsil and in the interfollicular area. Conversely, scoring of innervation was low in the germinal centre (*GC*) and mantle zone (*MZ*) of the lymphoid follicles and absent in the respiratory epithelium. If we compare the scoring of innervation in the connective tissue (*CT*) underlying the respiratory epithelium, we see that the scoring was higher in sheep older than 36 months. The statistical analysis applied to the results reveals that the increase of innervation is only influenced by age, regardless of genotype (scoring: *0* absence of labelled fibre (LF), *<1* less than 1 LF, *1* 1–5 LF, *2* 6–15 LF, *3* 16–30 LF, *4* 31–50 LF, *5* more than 51 LF)

Nerve markers	Tonsil compartments	Age and genotype			
		≤ 6 months		≥ 36 months	
		RES	SUSC	RES	SUSC
GFAP	GC	<1	1	1	1
	MZ	1	1	1	1
	Central axis	4	5	5	5
	Interfollicular area	4	5	5	5
	Sub-epithelial CT	1	1	4	3
	Respiratory epithelium	<1	0	<1	<1
NFH	GC	<1	1	1	2
	MZ	<1	<1	1	1
	Central axis	4	5	5	5
	Interfollicular area	3	4	4	4
	Sub-epithelial CT	1	1	3	2
	Respiratory epithelium	<1	<1	1	<1
NFL	GC	<1	<1	<1	<1
	MZ	<1	<1	<1	<1
	Central axis	3	3	3	4
	Interfollicular area	2	1	2	1
	Sub-epithelial CT	1	<1	2	1
	Respiratory epithelium	<1	0	1	0

were present (score: 4-5). Some of them were seen along and around the muscular wall of the blood vessels. The GFAP+ and NFH+ fibres were located in all the lymphoid compartments in variable proportions but only as sections of isolated fibres. They were abundant in the interfollicular area (score: 3-5), especially under and around the lymphoid follicles, their number tending to decrease towards the lymphoid tissues underlying the pseudostratified ciliary epithelium (Fig. 2a, b). The presence of nerve fibres inside the epithelium was rare (score: 0-1). Some sectioned NFH+ fibres were unusually observed inside the lymphoepithelial tissue.

The topographic partitioning of the NFL+ fibres was the same as that for the GFAP+ and NFH+ fibres; however, their number was definitely lower in each analysed compartment.

Analysis of the innervation pattern highlighted that the lymphoid follicles were poorly innervated by GFAP+, NFH + (score: <1-2) and NFL+ fibres (score: <1). When they were present, small sections of isolated fibres were detected in the GC and/or in the MZ of the follicles (Fig. 2c, d).

Taking into account all the pharyngeal tonsils analysed, the mean percentage of innervated GC was 3.15% for GFAP + fibres, 5.36% for NFH+ fibres and 0.80% for NFL fibres. However, in one sheep, which was younger than 6 months, the percentage of follicles innervated by NFH+ fibres was 25% higher than the mean (data not shown).

Regarding the innervation of the MZ without the presence of fibres in the GC, only 5.04% of the follicles contained GFAP+ fibres in this compartment, 3.47% contained NFH+ fibres and 0.56% contained NFL+ fibres.

Effect of age and genotype on innervation rate

Logistic regression tests were carried out on the results to determine the potential effect of age and genotype on the collected scoring of innervation and on the percentage of innervated GC and MZ.

Effect of age

The statistical analysis applied to the results of the semi-quantitative scoring of innervation in each compartment revealed that the number of GFAP+ and NFH+ fibres was only influenced by age with regard to the connective tissue underlying the respiratory epithelium. The scoring of innervation was significantly lower in the pharyngeal tonsils of sheep younger than 6 months when compared with sheep older than 30 months, regardless of genotype.

Taking into account the age of sheep within the groups of genotype resistant or susceptible towards scrapie, we observed that the percentages of GC and MZ innervated were lower in animals younger than 6 months when compared with sheep older than 36 months (Figs. 3, 4).

Effect of genotype

Considering the genotype only, even if the groups of sheep with the susceptible genotype seemed to have a higher percentage of innervation in the GC and MZ, the

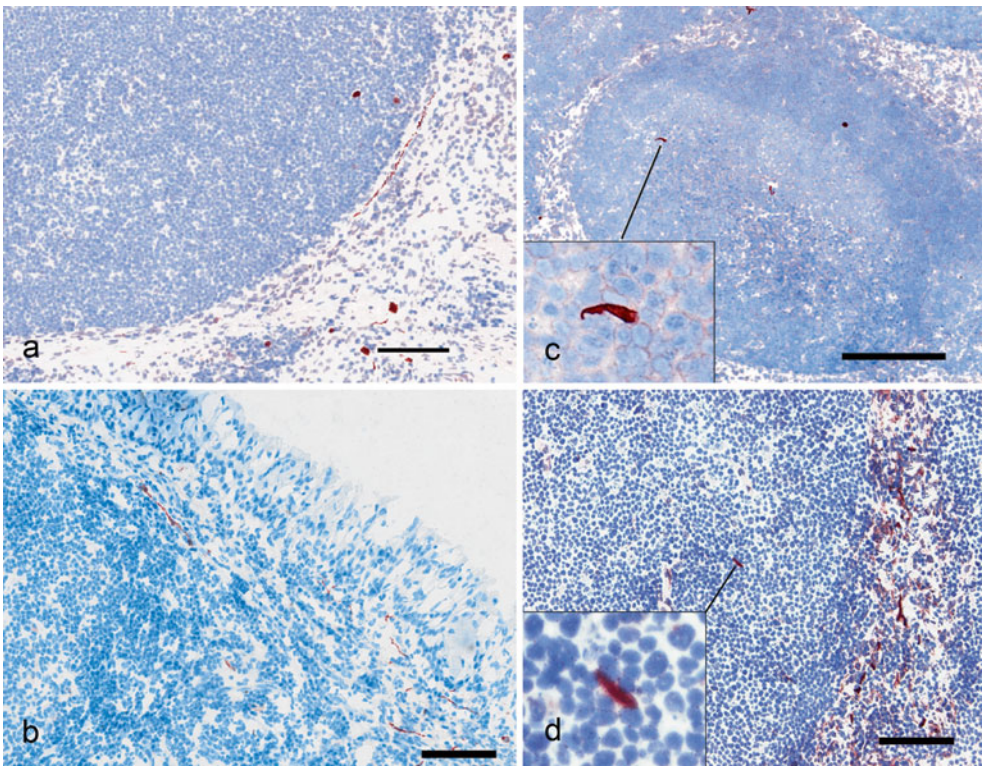

Fig. 2 Immunolabelling of ovine pharyngeal tonsillar cryosections by polyclonal antibodies (poAb): anti-neurofilament H (**a**, **c**) and anti-glial fibrillar acidic protein (**b**, **d**). Counterstaining with haematoxylin. **a** Abundant GFAP+ and NFH+ fibres were present in the loose collagenous connective tissue filling the central axis of the pharyngeal tonsil. Some of them followed the blood vessels. Isolated fibres ran through the interfollicular area, especially under and between the lymphoid follicles. *Bar* 100 μm. **b** GFAP+ fibres in the lymphoid tissues underlying the pseudostratified ciliary epithelium. *Bar* 100 μm. **c**, **d** NFH+ fibres (**c**) and GFAP+ fibres (**d**) labelled in the GC of lymphoid follicles. In this compartment, the fibre sections were rare, short and isolated. *Insets* Higher magnification view of indicated region. *Bar* 200 μm

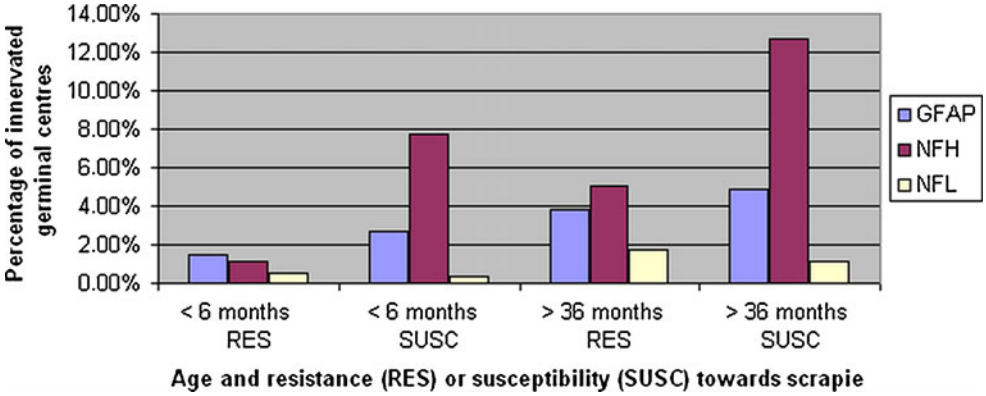

Fig. 3 Percentage of GC innervated by GFAP+, NFH+ or NFL+ fibres. Analysis of the innervation pattern highlighted that the lymphoid follicles were poorly innervated by GFAP+, NFH+ and NFL+ fibres (respectively, 3.15%, 5.36% and 0.80%). Whatever the genotype, the percentage of GC innervated was lower in animals younger than 6 months when compared with sheep older than 30 months. This difference, although small, was significant. Even if the groups of sheep with the susceptible genotype seemed to have a higher percentage of innervated GC than the groups of resistant genotype, the statistical analyses indicated that this difference was not significant

logistic regression demonstrated that this difference was not significant.

Neuroimmune interface

The topographic analysis of the innervation pattern revealed the presence of NFH+ and GFAP+ nerve fibres in the GC of the lymphoid follicles in which the FDCs were located. Therefore, we analysed, by confocal microscopy, double-fluorescent immunolabelling of FDCs and NFH+ or GFAP+ nerve fibres. The analysis of the spectra (red for FDCs and green for NFH+ or GFAP+ fibres) at the precise point of superimposition of these virtual colours (yellow point) revealed a perfect alignment of the peaks of intensity for each colour, thus establishing the ultra-close co-localisation between the cytoplasmic extensions of the FDCs and GFAP+ (Fig. 5) or NFH+ (Fig. 6) fibres present in the GC and/or in the MZ of some follicles. We could conclude that, even if the innervation rate inside the GC (respectively, 3.15% for GFAP+, 5.36% for NFH+ and 0.80% NFL+ nerve fibres) and the MZ (respectively, 5.04% for GFAP+, 3.47% for NFH+ and 0.56% NFL+ nerve fibres) was low, neuroimmune junctions between FDCs and NFH+ or GFAP+ fibres were detectable.

Discussion

Innervation pattern in pharyngeal tonsil: potential mechanism of neuroinvasion for PrPd

In natural scrapie, the most likely site of entry of the pathogen is generally accepted to be the oral route. Many authors have focused on the finding that, during the early phase of lymphoinvasion, the pathogen protein invades the gut mucosa through Peyer's patches and then invades the mesenteric lymph nodes, followed by the lymphoreticular system (LRS) unrelated to the digestive tract, e.g. the spleen (Maignien et al. 1999; Van Keulen et al. 2002). However, an increasing amount of data indicates that other starting points of infection are involved in prion pathogenesis, notably the respiratory system. Sheep and hamsters inoculated intranasally have been shown to develop TSE. Kincaid and Bartz (2007) have demonstrated that hamsters extranasally inoculated with a strain of transmissible mink encephalopathy agent develop the disease with an incubation period similar to that following oral inoculation. In a study dedicated to alternate ways of inoculation in sheep (Hamir et al. 2008), the authors have highlighted the presence of PrPd in lymphoid follicles of the pharyngeal tonsil

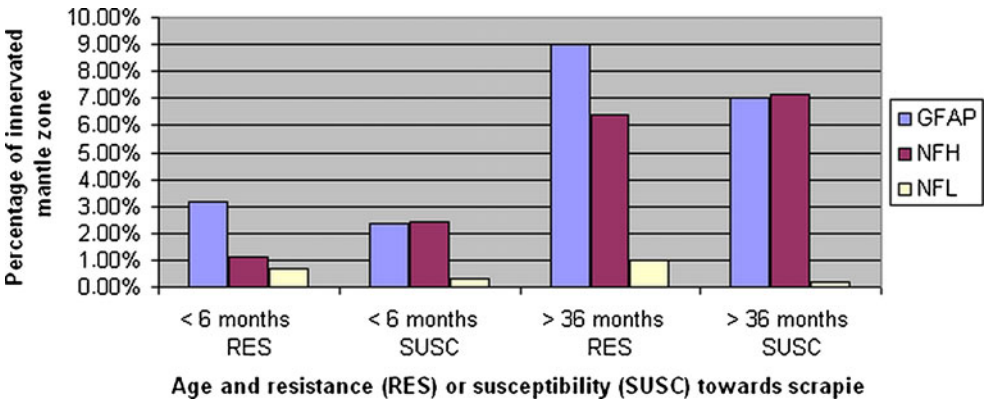

Fig. 4 Percentage of MZ innervated by GFAP+, NFH+ or NFL+ fibres. The percentage of MZ innervated by GFAP+, NFH+ or NFL+ fibres was low (respectively, 5.04%, 3.47% and 0.56%). Nevertheless, the statistical analyses revealed a significant increase in the number of innervated MZ, which was correlated with ageing but without influence of the genotype

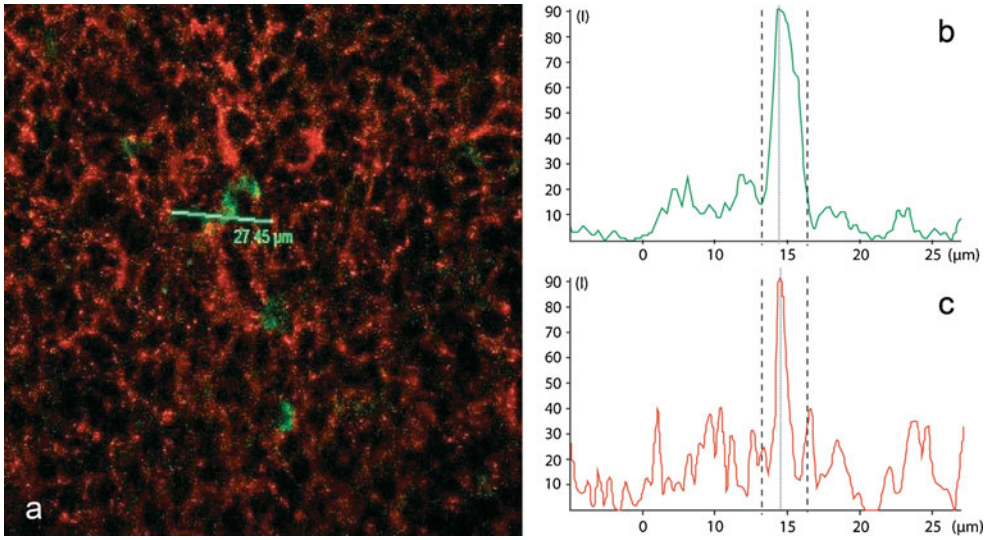

Fig. 5 Confocal analysis of co-localisation of FDCs and GFAP+ fibres within an ovine pharyngeal tonsil. Imaging (**a**) and spectral analysis (**b**, **c**) of close connections (*yellow*) between FDCs (*red*) and a GFAP+ fibre (*green*) immunostained, respectively, with FDC-B1 moAb and anti-GFAP poAb. The spectral study was carried out along the *green bar* of 27.45 µm in length traced on the selected confocal image. Superimposition of the *green* (**b**) and *red* (**c**) spectra gave a *yellow* signal. The spectral analyses revealed a correlation between the peaks of higher intensity in this *yellow* point indicating the close localisation of the FDC and the nerve fibre at this point

at 12 months after intranasal inoculation. This confirms that the pharyngeal tonsils represent a potential route for the scrapie agent dissemination.

In the lymph organs, the pathogenic agent replicates during a preclinical latent phase in the GC of the lymph follicles prior to reaching the CNS (Andreoletti et al. 2000; Heggebo et al. 2000).

Studies of mouse scrapie models have shown that mature FDCs are critical for replication in lymphoid tissues and, in their absence, neuroinvasion after peripheral challenge is significantly impaired (Brown et al. 1999; Mabbott et al. 2000).

The intrinsic innervation of lymphoid organs and contacts between FDCs and nerve fibres might function as a bridge between the TSE replicative machinery and the peripheral nervous system (Glatzel et al. 2001). As demonstrated in the mouse experimental model, the relative positioning of FDCs and nerve endings in the spleen controls the efficiency of peripheral prion infection (Prinz et al. 2003).

This study has focused on the innervation pattern inside the various compartments of the ovine pharyngeal tonsil taking into account that (1) young lambs appear to be more susceptible than adult sheep to oral TSE infection (Jeffrey and

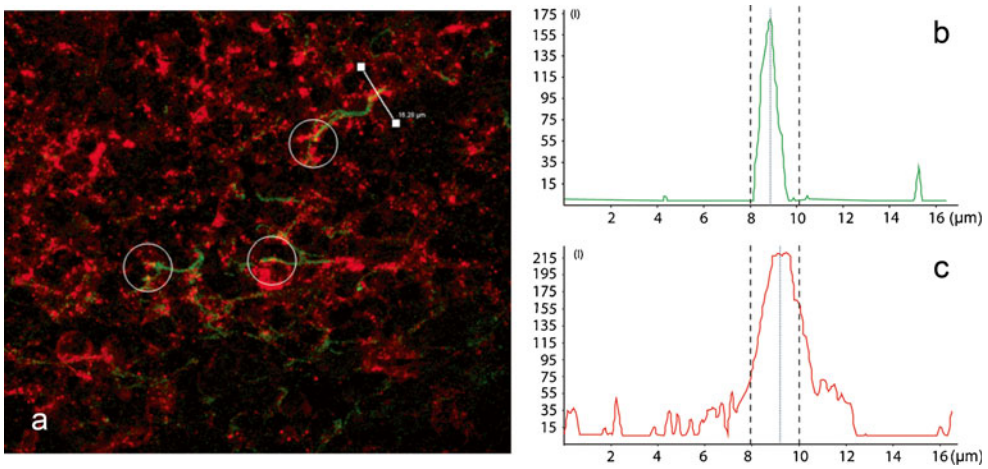

Fig. 6 Confocal analysis of co-localisation of FDCs and NFH+ fibres within an ovine pharyngeal tonsil. **a** Several close connections (*yellow*) between FDCs (*red*) and NFH+ fibres (*green*) are encircled (*white circles*). These structures were immunostained, respectively, with FDC-B1 moAb and anti-NFH poAb. **b**, **c** The spectral study was carried out along the *white bar* of 16.28 µm in length traced on the image. The correlation between the peaks of intensity at the *yellow* point revealed the close localisation of the labelled structures

Gonzalez 2007) and (2) the genotype influences the susceptibility or resistance towards scrapie (Baylis et al 2002).

High innervation in high traffic area versus poor innervation in PrPd replication centres

By immunohistochemical analysis, we have shown that nerve fibres are particularly abundant in the central axis of the tonsil, towards the interfollicular area and, in older sheep, in the connective tissue underlying the respiratory epithelium. Despite poor innervation of the follicular GC, which contains the FDCs and of the MZ, which contains the membrane extensions of the FDCs, morphological analysis has indicated neuroimmune junctions between FDCs and GFAP+ or NFH+ fibres. The presence of this neuroimmune interface supports the hypothesis of the transfer of pathogen prion protein through the "FDC-nerve synapse". However, as such physical connections are rare, this mechanism of neuroinvasion is unlikely to be unique. Other lymphoid compartments in the pharyngeal tonsil might also be sites of prion neuroinvasion. The T-dependent interfollicular area, which has the top scoring of innervation, contains many immune migratory cells, notably dendritic cells able to bind and carry the pathogen protein to the FDCs or directly to nerves endings, as demonstrated in the mouse model (Dorban et al. 2007). In older sheep, the loose connective tissue underlying the respiratory epithelium is rich in GFAP+ fibres and NFH+ fibres have been detected in the lymphoepithelial tissue in proximity to M cells involved in the uptake and transepithelial transport of pathogens (Casteleyn et al. 2010). Furthermore, the Schwann cells, revealed by the labelling of GFAP+ nerve fibres, are able to replicate PrPd in vitro and to transmit the disease to mice after intracerebral injection (Follet et al. 2002). They might be active in direct nerve infection. For these reasons, the interfollicular area, the connective tissue underlying the epithelium and the lymphoepithelium should be considered as key sites for the direct transfer of the pathogenic agents from the nasal cavity to the nerves endings that they contain or for neuroinvasion via mobile cells (macrophages and dendritic cells) after amplification in the GC.

These observations are corroborated by research on ovine Peyer's patches carried out by Marruchella et al. (2009) and McGovern et al. (2009). They have suggested that the rarity of FDC-nerve synapses in the lymphoid follicles of Peyer's patches throws doubt on the hypothesis that neuroinvasion is only the result of retrograde transportation of infectivity via nerves located in infected secondary follicles of the LRS.

No influence of genotype on innervation pattern

Many questions regarding the role of PrPc in the susceptibility to prions have been elucidated; however, the physiological role of PrP and the pathological mechanisms of neuroinvasion and neurodegeneration remain elusive (Flechsig and Weissmann 2004). Under physiological conditions, PrPc might notably play a role in neuronal and axonal development and in neurotogenesis through its interaction with laminin, a glycoprotein of the extracellular matrix, being involved in cell adhesion and proliferation (Cazaubon et al. 2007; Martins et al. 2001).

In sheep, the genotype at the *PRNP* gene and, subsequently, the intrinsic composition of the amino acids of the cellular prion protein influence the resistance/susceptibility towards scrapie. Since the nerves in the pharyngeal tonsil might be a portal of entry for prion neuroinvasion, the effect of genotype on the innervation pattern has to be evaluated.

In the present study, we have shown that the pattern of innervation is not affected by sheep genotype. Scrapie-free sheep with susceptible (ARR/VRQ or ARQ/ARQ) or resistant (ARR/ARR) genotypes exhibit the same distribution of GFAP+, NFH+ and NFL+ nerve fibres inside the various compartments of the pharyngeal tonsil. In view of our results, we can conclude that the innervation pattern is not modulated by the host *PRNP* genotype.

Differential innervation in relation to ovine age

In agreement with those of other studies (Ciriaco et al. 1995; Madden et al. 1997), our results indicate that the innervation of the lymphoid organs depends upon the age of the sheep. Sheep older than 30 months show an increase in the number of GFAP+ and NFH+ nerve fibres in the connective tissue underlying the respiratory epithelium when compared with lambs. Similarly, Maruchella et al. (2009) have reported an increase in the number of PgP9.5+ nerve fibres in the dome area of ovine Peyer's patches underlying the intestinal epithelium in old sheep and this shows no correlation with the genotype. In both cases, and increase occurs in innervation in areas close to the site of entry of PrPd with ageing.

GC from Peyer's patches of bovines older than 24 months contain numerous NFL+ and NFH+ nerve fibres in contrast to GC of calves, which are poorly innervated (Defaweux et al. 2007).

In sheep, we have illustrated an increase with ageing of the number of GC and surrounding MZ innervated by GFAP +, NFH+ and NFL+ nerve fibres. In the case of scrapie, the pathogenic relevance of the innervation pattern increasing with ageing is questionable, as sheep are frequently observed to be contaminated soon after birth or at a young age (Jeffrey and Gonzalez 2007). A possible explanation for this is that, as neuroinvasion occurs in the advanced phase of infection after replication of the pathogen in the lymphoid follicles, the denser network of nerve fibres inside and around the centres of amplification of PrPd observed in older sheep might ensure more efficient infectivity.

Concluding remarks

In conclusion, our results suggest that the transfer of prion infectivity through "FDC-nerve synapses" remains plausible, as we have demonstrated connections between both of these structures. However, the poor innervation of the lymphoid follicles supports other mechanisms of neuroinvasion, such as "dendritic cells–nerve synapses", or haematogenous or lymphatic routes.

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