Investigation of close interactions between sympathetic neural fibres and the follicular dendritic cells network in the mouse spleen

C. Demonceau,[1] A.S. Marshall,[2] J. Sales,[3] E. Heinen[1]

[1]Institute of Human Histology, Immunology Centre, CRPP, University of Liège, Liège, Belgium;
[2]Neuropathogenesis Unit, Institute for Animal Health, Ogston Building, Edinburgh, United Kingdom;
[3]Biomathematics & Statistics Scotland, Edinburgh, United Kingdom

In this study, co-localization between sympathetic neural fibres and the follicular dendritic cells (FDCs) network was observed within the mouse spleen by confocal technology. Immunohistochemical techniques were used to reveal the rare interactions between the FDCs network and sympathetic neural fibres. We estimated the frequency of three kinds of close interactions which could be defined as overlaps, contacts or neural fibres closer than 10 μm from a FDCs network. Using these estimates, a comparison was made between five uninfected mouse strains exhibiting the same *Prnp[a]* genotype but showing different incubation periods when inoculated with primary bovine spongiform encephalopathy (BSE)-infected brain. In prion disease, infectivity is generally detected in the spleen much earlier than in the brain, especially after peripheral inoculation. The way by which the infectious agent reaches the central nervous system is still unclear. From the five mouse strains, we obtained differences in the proportion of splenic FDCs networks with close interactions. Our work suggests that the percentage of splenic FDCs networks with at least one sympathetic neural fibre in close vicinity may influence the length of incubation period.

Key words: Follicular dendritic cell, sympathetic nervous system, incubation period, prion.

Correspondence: Caroline Demonceau,
Institute of Human Histology, Immunology Centre,
CRPP, University of Liège, 1 Avenue de l'Hôpital,
4000 Liège, Belgium
Tel.: +32.4.366 5171.
Fax: +32.4.366 4321.
E-mail: cdemonceau@student.ulg.ac.be

Transmissible spongiform encephalopathies (TSEs), also called prion diseases, are fatal progressive neurodegenerative disorders of man and animals affecting the central nervous system (CNS). There is quite a large number of different prion strains, which can be distinguished in experimentally infected laboratory animals by particular durations of the asymptomatic incubation period and peculiar neuropathology profiles in inbred mouse strains (Bruce *et al.,* 1991). For transmission of bovine spongiform encephalopathy (BSE) from cattle to mice, a replication phase in the spleen appears to be obligatory after peripheral infection before neuroinvasion can occur; this seems to be the case even when infection is introduced directly into the brain (Bruce *et al.,* 1994). The intracerebral route is not faster than the intraperitoneal route and a peripheral processing step involving mature FDC may facilitate spread of BSE infection to the CNS (Brown *et al.,* 1997).

Several lines of evidence indicate an involvement of the lymphoreticular system (LRS) in the development of prion diseases. After peripheral inoculation prions usually replicate in host's LRS tissues before neuroinvasion occurs (for review see Aguzzi & Heikenwalder, 2006; Mabbott & MacPherson, 2006). The key pathogenetic event in all animal and human TSEs is represented by the accumulation in the CNS, as well as in a number of peripheral nervous and LRS tissues, of a disease-specific PrP isoform (PrPd) of the normal host-encoded *cellular prion protein* (PrPc), with the incubation period and the clinico-pathological disease phenotype depending upon the host's PrP genotype and the prion strain (Farquhar *et al.,* 1994; Farquhar *et al.,* 1996). PrPc expression in peripheral tissues from both uninfected and infected mice is generally located on follicular dendritic cells (FDC) within lymphoid follicles, where it appears critical for prion replication and translocation to nerves (Kitamoto *et al.,* 1991; McBride *et al.,* 1992; O'Rourke *et al.,*

1994; Fraser *et al.,* 1996; Klein *et al.,* 1997; Klein *et al.,* 1998; Brown *et al.,* 1999; Mabbott *et al.,* 2000; Raeber *et al.,* 2001; Aguzzi & Heikenwalder, 2006; Mabbott & MacPherson, 2006). That FDC may be critical for prion infection pathogenesis in most species is also suggested by the presence of PrPd deposits in LRS tissues from patients with the *variant* Creutzfeldt-Jakob disease (vCJD; Hill *et al.,* 1999) and from scrapie-affected sheep (van Keulen *et al.,* 1996). However, FDC are non-migratory and how prion infection translocates into the peripheral and central nervous system is unknown (Mabbott and Bruce, 2003).

Evidence is accumulating on the role of the sympathetic nervous system (SNS) in neuroinvasion in several natural and experimental prion diseases. The sympathetic nodes of hamsters orally infected with scrapie accumulate PrP^{Sc} (McBride and Beekes, 1999). Sympathectomy delays intraperitoneally routed scrapie in mice, and sympathetic hyper-innervation accelerated both replication within the spleen and neuroinvasion (Glatzel *et al.,* 2001). Lymphoid organs are densely innervated by the SNS and there is evidence for direct interactions between noradrenergic fibres and lymphocytes and macrophages (Felten *et al.,* 1985; Felten *et al.,* 1987; Felten and Felten, 1988; Felten and Olschowka, 1987; Straub, 2004). Noradrenergic fibres have also been observed close to FDC in healthy sheep spleen, and to PrP^{Sc}–accumulating cells in sheep affected by natural scrapie (Bencsik *et al.,* 2001a; Bencsik *et al.,* 2001b). A recent transgenic approach co-locating FDC with splenic nerves reduced the incubation period of intraperitoneally inoculated RML scrapie in mice, suggesting faster transfer into the central nervous system when sympathetic nerve endings are in the vicinity of FDC network (Prinz *et al.,* 2003).

A non-transgenic model was used to investigate the sympathetic innervation of splenic FDC networks. As the incubation period is under the influence of the host *Prnp* genotype, the strains were chosen to have the same *Prnp^a* gene allele to avoid any genetic effects of this gene on the incubation periods; *Prnp*, the gene that encodes for cellular prion protein, has two main variants in mice that differ only by their amino acids at coding positions 108 and 189 (Moore *et al.,* 1998). Five mouse strains, that exhibit different incubation periods when inoculated with primary BSE, were included in the study. The three RIII-2, RIII-1 and 129/Ola

mouse strains have equivalent incubation periods which are 100-days shorter than those of C57BL and C57BL/6 (=B6) mouse strains: a mean incubation period of approximately 315 days compared to approximately 425 days for the C57BL strains. There is no evidence that these incubation periods have changed over the period since they were first reported (Bruce *et al.,* 1994; Bruce *et al.,* 1997; Fraser *et al.,* 1992; Green *et al.,* 2005; Manolakou *et al.,* 2001; C. Farquhar, personal communication).

Materials and Methods

Animals

All five *Prnp^a* mouse strains studied (RIII-1, RIII-2, 129/Ola, C57BL, and C57BL/6 (subsequently referred to as B6) were bred under SPF conditions (except for RIII-1 which were maintained in quarantine), at the Neuropathogenesis Unit (NPU), Institute for Animal Health. For each mouse strain, 6 females (10 weeks old ± 2 days) were sacrificed by cervical disruption and the spleen dissected. RIII-1 and RIII-2 are two different importations of the same mouse strain, but kept in different conditions due to welfare provisions. All protocols involving experimental animals were submitted to the Local Ethical Review Committee for approval and were performed under licence in accordance with the UK Home Office Regulations (Scientific Procedures) Act 1986.

Immunohistochemistry

Spleens were immersed in freshly prepared PLP (periodate-lysine-paraformaldehyde) fixative (0.0375 M phosphate buffer pH 7.4, 2% paraformaldehyde, 0.1 M sodium m-periodate, 0.075 M D L-lysine monohydrochloride) for six hours. Longitudinal slices of 100 μm thickness were sliced with a vibratome (Oxford Vibratome) and kept in 0.05 M Sörensen phosphate buffer, pH 7.4 at 4°C into wells of a labelled 24-well plate until the next day.

For each spleen from each mouse, double-staining was performed upon three thick 100 μm spleen slices: the 8th, 11th and 14th sections from the concave side are representative of the whole mouse spleen. In general, sections 8 and 14 were at the extremities of the spleen with section 11 placed centrally within the spleen; sections 1 to 7 were extremely small

and narrow. The total number of sections per spleen was between 15 and 16 sections with the last one sticking to the vibratome support. Immuno-histochemistry was performed at room temperature and all solutions were prepared with Tris buffered saline (7.45 g Tris pH 7.6, 2 g BSA, 8.766 g NaCl in 1 l distilled water). After blocking for 20 min with normal goat serum (1:20, Dako), floating sections were incubated overnight with a polyclonal anti-tyrosine hydroxylase antibody (TH, 1:200, Chemicon) to identify sympathetic neural fibres, before washing, and incubating with a secondary detection anti-rabbit Alexa488 labelled antibody (15 µg/mL, BD Pharmingen) for one hour. To detect FDC networks, sections were incubated with mono-clonal FDC-M2 biotinylated antibody (1:400, ImmunoKontact) for two hours. The sections were rinsed and incubated with streptavidin Alexa594 (2.5 µm/mL, BD Pharmingen) for one hour. Rinsed floating sections were delicately transferred onto slides and mounted with mounting fluorescent medi-um (Dako). Slides were then left to dry at 4°C overnight, coverslipped and sealed with nail varnish.

Confocal analysis and morphometric measurements

Analysis was performed upon vibratome-sec-tioned spleen tissue using a Zeiss LSM 5 Pascal confocal attached to a Zeiss Axioskop 2 MOT fluo-rescent microscope. All spleen sections were observed blind by only one investigator and the laser was retained at the same settings during analysis performed in this study. The z-stack series were recorded at x63 magnification lens with a 0.5 µm gap between each confocal scan, and the screen resolution determined that one pixel corresponded to 0.285 µm. Only the two external 10 µm z-stack series scans, from each 100 µm-thick spleen section were analysed, as the FDC-M2 antibody used did not label throughout the section. In each confocal scan of the z-stack series, the counts of three kinds of close interactions between one tyrosine hydroxy-lase positive (TH$^+$) neural fibres labelling and one individual extension from FDC network labelling were performed: the number of overlaps (pixels of both colours superimposed and thus appear as a yellow colour), contacts (pixels of both red and green colours adjacent to each other) and TH$^+$ neu-ral fibres closer than 10 µm from FDC network staining were counted for each spleen section. This was achieved by firstly observing the whole section at x40 magnification and the FDC network and TH

staining pattern noted. In areas where FDC network and TH labelled cells were in proximity the section was then scanned with the x63 magnification lens. The Pascal software was used to measure distances and analysed colour superposition to confirm the close interactions observed. Morphometric meas-urements of area were performed with the Image-Pro® PLUS software attached to a Zeiss fluores-cent microscope.

For each of the three thick sections of the spleen per mouse, measurements were made of: the spleen area, the area of FDC network (as measured in the first confocal scan of the z-stack series from each side of the 100 µm thick section), the number of FDC networks, and the number of neural fibres in close interactions with each FDC network for each confocal scan of the 10 µm z-stack series. The num-ber of overlaps between FDC network and neural fibres, the number of contacts between FDC net-work and neural fibres, and the number of neural fibres closer than 10 µm from the FDC network were recorded separately. For each spleen section analysed, the spleen volume and the FDC networks volume analysed were estimated by multiplying respectively the area of the spleen section and the area of FDC networks inside this same spleen sec-tion by 10 µm thickness of the z-stack series.

Statistical methods

The number of interactions between FDC net-works and neural fibres was modelled using a gen-eralised linear mixed model (GLMM) with a Poisson error and log link function. Individual analyses for each type of close interaction (over-laps, actual contacts or neural fibres closer than 10 µm from FDC network) and an overall analysis of the total number of close interactions were per-formed. The models fitted for each count had mouse strain, section and strain x section interaction effects as fixed effects and the mice within strain as random effects. The dispersion parameter was esti-mated in the model as it would be expected that the method of counting would result in the data being over-dispersed. The proportion of FDC networks with neural fibres was modelled in the same way as the number of interactions except that a binomial distribution and logit link function were used in the GLMM. The percentage of spleen volume occupied by FDC networks was analysed using a general lin-ear mixed model. Although differences in strains were observed for several of the variables of inter-
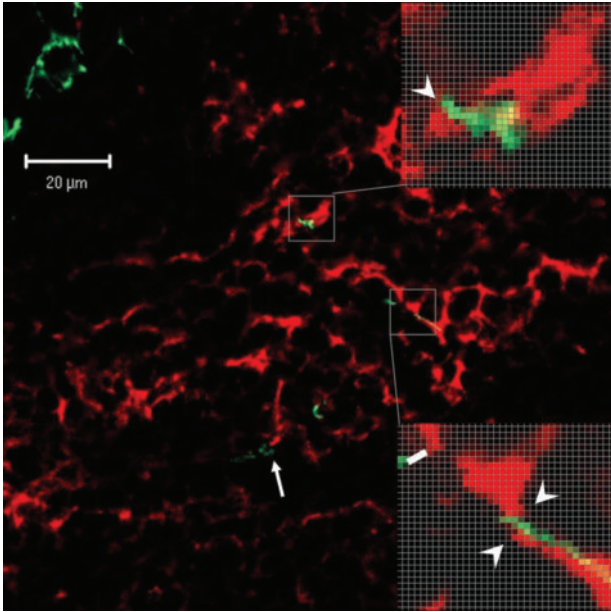
Figure 1. Confocal laser scanning microscopy of double immuno-fluorescent labelling with TH (green) and FDC-M2 (red) antibod-ies. We observed one confocal scan from a z-stack series show-ing the three types of close interactions: overlap (yellow colour from co-localization of green and red colours), contact between TH+ neural fibres and FDC network (red and green colours side by side), and TH+ neural fibres closer than 10 µm from FDC net-work. In the top left we observed a tubular-like structure asso-ciated with TH+ labelling. The top right pixelbox shows a select-ed area where both yellow co-localization on the right and con-tact (arrowhead) with another FDC network labelling on the left are enlarged. The bottom right pixelbox shows a selected area where there are one yellow co-localization in the bottom right, two contacts (arrowheads) with different FDC network labellings in the centre, and one TH+ neural fibre closer than 10 µm from FDC network in the top left (large white bar represents 0.86 µm). The arrow points to a TH+ neural fibre surrounding the FDC network.
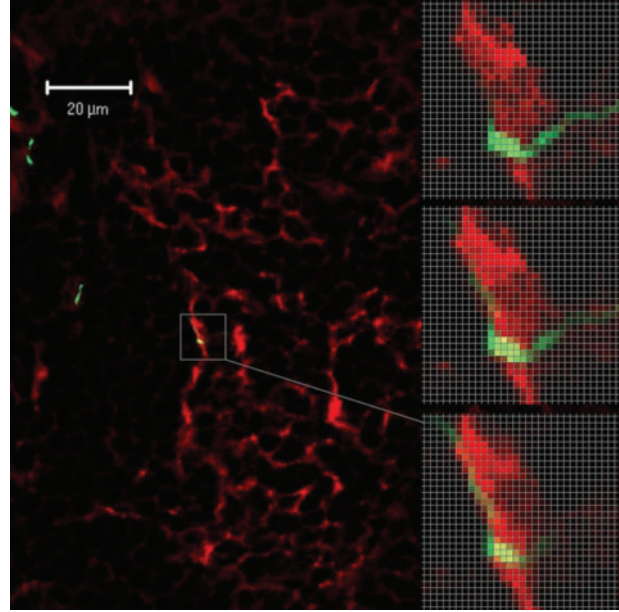

Figure 2. Confocal laser scan of double immunofluorescent labelling with TH (green) and FDC-M2 (red) antibodies. We observed one confocal scan from a z-stack series showing an overlap (yellow) between TH+ neural fibres (green) and FDC net-work (red). The bottom right pixelbox shows the selected area where yellow co-localization is enlarged. The upper pixelboxes are the two preceding serial confocal scans of the same area showing co-localization and contact respectively. Gap between confocal scan = 0.5 µm.

est there did not appear to be any appreciable effect of section. Also, a similar pattern of results was obtained by looking at each type of close inter-actions separately or by looking at the total number of close interactions, consequently only results for total close interactions are reported; these are expressed as strain means with associated standard errors estimated from the simplified statistical models. Analyses were carried out using Genstat 8th Edition (VSN International Ltd).

Results

Confocal analysis of spleen specimens examined revealed close interactions between sympathetic neural fibres and FDC network in all of them. Most of TH+ neural fibres appeared to be associated with

structures of tubular morphology with some projec-tions going to the FDC network. Close interactions occasionally occurred at this interface and some-times some neural fibres surrounded the FDC net-work (Figure 1). Figure 2 shows overlap between TH+ neural fibres and FDC network (yellow pixels from co-localization of green-TH[+] and red-FDC network labels of the confocal scan). The bottom right pixelbox (Figure 2) represents a zoom of this overlap and the upper pixelboxes are the two pre-ceding serial confocal scans of the z-stack series. It shows how a contact (pixels of both colours adja-cent to each other) can move through a z-stack series to become an overlap at the second and third confocal scans 0.5 and 1 µm away.

The number and the volume of FDC networks

Both within and between strains, there was a strong positive linear relationship between the num-ber of FDC networks per unit volume of spleen and the percentage of spleen volume occupied by FDC networks (*data not shown*). Three mouse strains, RIII-2, RIII-1 and B6, showed the highest number of FDC networks (2.4, 2.2 and 2.4/10[7] µm[3] spleen

respectively) and the highest percentage of volume occupied by FDC network (5%, 5.4% and 3.4% respectively). The lowest values were observed for the 129/Ola mouse strain with 1.0 FDC networks/10^7 µm³ spleen and 1.9% FDC network, and the C57BL mouse strain having intermediary values with 1.8 and 2.8% respectively (Table 1).

The number of close interactions

To estimate how much the FDC networks were sympathetically innervated between the five mouse strains, three kinds of close interactions were counted through the confocal scans of each z-stack series: overlaps, contacts and TH+ neural fibres located closer than 10 µm from a FDC network which are all represented in Figures 1 and 2.

There were differences in the mean number of close interactions between the different strains. With reference to FDC network per unit volume, RIII-2 and 129/Ola mouse strains showed the highest number of close interactions with 31.1/10^7 µm³ FDC network and 32.0/10^7 µm³ FDC networks respectively; the three RIII-1, C57BL and B6 mouse strains having a lower number of close interactions with 13.6, 17.8 and 18.7 /10^7 µm³ FDC network respectively (Table 1). However, the mean number of close interactions, in FDC networks that had at least one close interaction, was very similar for all the five mouse strains and averaged about 4 close interactions per FDC network with close interactions (Table 1).

The proportion of FDC networks with close interactions

There was strong evidence that the proportion of FDC network that had at least one close interaction between TH+ neural fibres and FDC network differed between strains ($p < 0.001$). The estimated percentages from the statistical fitted model are shown in Table 1. The RIII-2 strain had the highest estimated proportion with approximately 15% of FDC networks with close interactions between sympathetic neural fibres and FDC network whereas the C57BL and B6 strains had only 6%; RIII-1 and 129/Ola mouse strains had intermediary percentages with about 9% and 12% respectively (Table 1).

Discussion

In this study, the focus was on close interactions between sympathetic neural fibres and FDC networks in mouse spleen. An immunohistochemical approach was performed by means of confocal technology to have an overview of this neuroimmune interface within the spleen of five mouse strains which show different incubation periods when inoculated with primary BSE. No mouse strain which exhibits increased sympathetic innervation, for example K14NGF transgenic mouse strain, was included in this study as the incubation period after inoculation with primary BSE is unknown.

There appeared to be no association between prion incubation periods in the different mouse strains and the density of FDC networks, or the percentage of splenic volume occupied by FDC networks (analyses involving the number of FDC networks or the volume of FDC networks gave similar informations). Three mouse strains, RIII-2, RIII-1 and C57BL/6, have approximately 2.4 FDC networks/10^7 µm³ spleen; the two first strains exhibit a *short* incubation period whereas the C57BL/6 has a *long* incubation period. These results suggest that the FDC network hypertrophy is not a pre-required

Table 1. Means and (standard errors) estimated from fitting generalised linear mixed models to the data. RIII-2, RIII-1, 129/Ola mouse strains exhibit *short* incubation periods when infected with primary BSE; C57BL and B6 show *long* incubation periods after primary BSE infection.

Mouse strain	RIII-2	RIII-1	129/Ola	C57BL	B6
Number of FDC networks per 10^7 µm³ spleen volume	2.40 (0.29)	2.18 (0.29)	1.03 (0.14)	1.75 (0.23)	2.38 (0.28)
Volume of FDC network as a percentage of spleen volume	5.00 (0.38)	5.37 (0.41)	1.86 (0.39)	2.77 (0.39)	3.39 (0.38)
Number of close interactions per 10^7 µm³ volume of FDC network	31.1 (4.0)	13.6 (2.6)	32.0 (7.0)	17.8 (4.0)	18.7 (2.8)
Number of close interactions per number of FDC networks with close interactions	4.23 (0.53)	3.74 (0.61)	4.18 (0.73)	4.25 (0.75)	4.26 (0.58)
Percentage of FDC networks with close interactions	15.4 (1.9)	9.0 (1.5)	11.6 (2.2)	6.4 (1.3)	6.2 (0.9)

for a more effective neuroinvasion. This observation corresponds with that of Prinz and co-workers' where, before or after RML prion strain infection, no relationship between FDC network hypertrophy and a shorter incubation period was observed (Prinz *et al.,* 2003). Nonetheless, as hypertrophy of FDC dendrites was previously observed in C57BL mice infected with ME7 prion strain (McGovern *et al.,* 2004), a similar phenomenon could be also induced during primary BSE infection in these five mouse strains, thus putatively exerting an effect upon neuroinvasion.

In regard to innervation, from all splenic lymphoid follicles examined in the five mouse strains, we generally observed sympathetic neural fibres associated with structures of tubular morphology, certainly the central artery, with thin neural fibres radiating away as described previously (Felten *et al.,* 1985). Some neural fibres were also observed surrounding the FDC networks in lymphoid follicles, probably located at the mantle zone. For the first time, it was demonstrated that co-localization does occur between FDC networks and sympathetic neural fibres in the mouse spleen. This finding is in contrast to a previous study by our group that demonstrated that germinal centres of C57BL/6 mouse strain, which contained FDC networks, are not innervated in Peyer's patches, another peripheral lymphoid tissue (Defaweux *et al.,* 2005). However, our finding is supported by the work of Heggebø *et al.* in which Peyer's patches lymphoid follicles from Suffolk sheep were shown to harbour a more or less prominent nervous fibres (PGP9.5[+]) network, having close interactions with both FDC and so-called *tingible body macrophages* (Heggebø *et al.,* 2003). Electronic microscopy and immunohistochemical studies would need to be combined to identify what kind of junction occurs between nerve endings and a dendrite characterized as coming from a FDC network.

In order to estimate FDC networks sympathetic innervation within the spleen of the five mouse strains, three kinds of close interactions between sympathetic neural fibres and FDC networks were counted in each scan of the 10 µm z-stack series: overlaps (yellow confocal pixels from superposition of both red and green colours), contacts (confocal pixels from both red and green colours side by side) and TH[+] neural fibres closer than 10 µm from FDC network. The method used gives evidence of how the TH[+] neural fibres interact with FDC networks in the

volume of the spleen. Of particular interest was whether the number of these close interactions differed between the five mouse strains and whether any observed difference was related to the known differences in incubation period when inoculated with primary BSE.

The counts of close interactions gave information about the nature of the interface between sympathetic neural fibres and FDC networks. The RIII-2 and 129/Ola mouse strains showed the highest number of close interactions per volume of FDC network, followed by the B6, C57BL and RIII-1 mouse strains.

For the five mouse strains, the mean number of close interactions for FDC networks with any close interactions appeared similar and averaged around four per network. Close interactions between TH neural fibres and FDC networks seemed to cluster within a few FDC networks as the same nerve could be reported several times throughout the confocal z-stack series. A 3D-structure would be needed to observe precisely where contacts occurred and to quantify the interface area between FDC and sympathetic neural fibres.

Of particularly interest was the number of FDC networks that had at least one close interaction compared to the total number of FDC network. The findings indicate that this proportion varies between mouse strains. The three mouse strains (RIII-2, RIII-1 and 129/Ola) that have the highest proportions are also those that exhibit a shorter incubation period when inoculated with primary BSE. Rather than the actual number of neural fibres in close proximity to FDC network as proposed by Prinz *et al.* (2003), our work suggests that the percentage of FDC network with close interactions between sympathetic neural fibres and the FDC network may increase the possibility of neuroinvasion and thus cause a shorter incubation period in specific mouse strains.

With regard to prion diseases, the FDC seem to be implied in infectious agent replication before neuroinvasion by the sympathetic nervous system. Even the putative role of different PrP[c] expression levels are supposed, as well as, the multiple quantitative trait loci linked to differences in the incubation period (Lloyd *et al.,* 2001); the aim of this study is to gain a better understanding of the additional/complementary role of the sympathetic nerve fibres/FDC networks interface. Spleens of five mouse strains with the same *Prnp*[a] genotype, but

exhibiting different incubation periods when inoculated with primary BSE, were studied. Neither the mean density nor the volume of FDC networks for the different strains appeared to be related to the incubation periods. As FDC do not migrate, they cannot account for the transport of infectivity from the lymphoreticular system to the peripheral nervous system. However, real co-localizations do occur between TH neural fibres and FDC networks, and the number of close interactions could play a role in the time of access of prion agent to the CNS, and hence affect the incubation periods. Moreover, even if any immunostain of PrP^{Sc} could lead to the idea of a translocation by other cells (unlikely with regard to data from Raymond and Mabbott, 2007), our observation is still relevant compared with the theory of FDC-reservoir of PrP^{Sc} before spreading to sympathetic neural fibres. There is some evidence from this study that the proportion of FDC networks with close interactions may influence the incubation period but further work is needed to investigate this possibility.

Acknowledgements

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