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REGULAR ARTICLE

Involvement of astrocytes in transmissible spongiform encephalopathies: a confocal microscopy study

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Abstract Astroglial proliferation associated with pathological prion protein (PrPsc) deposition is widely described in Transmissible Spongiform Encephalopathies (TSEs). However, little is known of the actual role played by glia in their pathogenesis. The aim of the study has been to determine whether PrPsc is located exclusively in neurons or in both neurons and glial cells present in the central nervous system in a natural Scrapie model. Samples of cerebellum from 25 Scrapie sheep from various flocks were sectioned. Following epitope retrieval with formic acid, proteinase K and heat treatment, primary antibody L42 and primary antibodies against glial fibrillary acidic protein were applied as prion- and astrocytic-specific markers, respectively. For visualization, a suitable mixture of fluorochrome-conjugated secondary antibodies was used. Relevant controls were processed in the same manner. As determined by confocal microscopy, PrPsc deposits co-localized with glial cells in all samples. Our results suggest that these cells can sustain active prion propagation, in agreement with similar findings from other studies of primary cell cultures and inoculated mice. Furthermore, despite ongoing debate regarding whether varied TSE sources show differences in their tropism for different cell lineages in the brains of affected animals, no differences in co-localization results were seen.

Keywords Glia · Astrocytes · Prion protein · Confocal microscopy · Scrapie · Sheep

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Introduction

As noted in Ye et al. (1998b), the histopathological changes typically found in the brains of Scrapie animals (the prototype of the fatal Transmissible Spongiform Encephalopathies; TSEs), are vacuolization and neuronal and neurite degeneration, the deposition of pathological prion protein (PrPsc; Kretzschmar et al. 1986) and gliosis (microgliosis and astrocytosis; Kretzschmar et al. 1986).

Initially, PrPsc appeared to be primarily localized to neuronal cell bodies. However, it could also be found in the neuropil outside these neuronal bodies in terminal stages of the disease, giving the impression that it was located in astrocytes or astrocytic processes (Kretzschmar et al. 1991). Furthermore, several studies have demonstrated that, in some breeds affected by natural Scrapie, such as Suffolk sheep, even higher concentrations of PrPsc deposits associated with glia are present, especially in astrocytes (González et al. 2003). For this reason, many studies have investigated the connection between PrPsc and glial cells, especially astrocytes, with respect to TSEs (Moser et al. 1995).

As it is moderately well accepted, the conversion of cellular prion protein (PrPc) into PrPsc occurs in neurons, based on studies demonstrating that the cellular isoform displays a predominantly neuronal localization (Mironov et al. 2003). Moreover, neurons express prion protein mRNA (Kretzschmar et al. 1986) and initially synthesize the pathological isoform (Safar et al. 1990). Additionally, intraneuronal immunostaining has

been observed that resembles the PrPsc deposits observed in the development of clinical disease. Even though several studies have corroborated the claim that neurons are the first cells affected, they have also shown abnormal accumulation around and associated with astrocytes. However, during the initial disease course, they display only reactive changes and no damage is seen (Jeffrey et al. 2004). These astrocytes have also been hypothesized to generate PrPsc, which could help to promote the astrocytic proliferation of PrPsc (Ye et al. 1998b), because this protein has been seen to accumulate in glial cells before the appearance of neuropathological changes in Scrapie (Diedrich et al. 1991). Electron microscopy studies with colloidal gold have not detected disease-specific prion protein (PrPd) in or around neuronal cell bodies. However, PrPd has been detected around astrocytes (Jeffrey et al. 2004). One hypothesis contends that, although the normal cellular isoform in nervous tissue is directly associated with neurons (Kretzschmar et al. 1986), PrPsc accumulates in astrocytes (Diedrich et al. 1991). However, this hypothesis is still pending clarification.

The demonstration of cellular accumulations of PrPsc in astrocytes and the documentation by the in situ hybridization of mRNA encoding this protein in astrocytes are compatible with the notion that the Scrapie agent replicates in astroctyes and induces the conversion of PrPc to PrPsc (Diedrich et al. 1991), although the deposits precede this process and neuronal loss (Moser et al. 1995). On the other hand, astrogliosis, defined by an accumulation of glial fibrillary acidic protein (GFAP) mRNA (Moser et al. 1995), is a common characteristic lesion of this group of diseases (Ye et al. 1998a), even preceding the pathological (Georgsson et al. 1993) and spongiform (Manuelidis et al. 1987) changes. This finding is more evident in the cerebellum in which the increased expression of GFAP coincides with high concentrations of the agent before the occurrence of pathological lesions (Manuelidis et al. 1987). Thus, astrocytosis seems to be caused directly by the infectious agent, rather than as a consequence of secondary neuronal damage (Georgsson et al. 1993). As some studies have suggested, all of these processes could be caused by PrPsc acting as a factor in astroglial cellular proliferation, such as the α-transforming growth factor (α-TGF) produced by brain cultures exposed to Creutzfeldt-Jakob disease (CJD) material, which stimulates the expression of GFAP (Oleszak et al. 1989; Poleshchuk et al. 1990). Several studies support this hypothesis, suggesting that astrocyte activation is caused by PrPsc (Lasmézas et al. 1996), because it is a factor responsible for the activation of astrocytic gene expression (Diedrich et al. 1991). Meanwhile, other authors have postulated that astrogliosis induces the conversion of PrPc to PrPsc (Thellung et al. 2000), because reactive astrocytes have the capacity to produce and accumulate PrPsc (Ye et al. 1998b). This proposal is in accordance with the finding that astrocytes are target-cells for the causative agent of human CJD and animal TSEs (Lefrançois et al. 1994), promoting the formation and possibly the replication, of the agent (Diedrich et al. 1991; Georgsson et al. 1993). Nevertheless, the actual role of astrocytes in prion propagation has not been precisely described.

On the other hand, the distribution of PrPsc in neuronal tissue (Ferrer et al. 2000) has been widely studied. Indeed, the PrPsc distribution profiles obtained by specific immunohistochemistry allow the differentiation of TSE sources (González et al. 2002, 2005; Bencsik et al. 2007) assuming that these specific patterns are primarily influenced by a combination of both the source of the agent (González et al. 2003) and the genotype corresponding to the host (González et al. 2002). Recent studies have led to the proposal that this phenotype is partly the result of differences in the relative affinity of different agents for different cell types and of differences in the cellular processing of PrPsc (DeArmond et al. 1997). Therefore, different strains might preferentially target some cell populations, giving rise to, for example, a more prominent neuron-associated rather than glia-associated pattern of PrPsc deposition (Jeffrey et al. 2009). These differences might also explain the possible variations in the tropism between the classical and atypical Scrapie strains (Moore et al. 2008).

Thus, with the main aim of clarifying the involvement of astrocytes in TSE pathogenesis, the present study has employed confocal microscopy to determine whether PrPsc co-localizes with astrocytes in the cerebellum of a natural Scrapie model. In addition to this primary aim, our secondary objective has been to assess whether differences in this co-localization exist that are dependent on the source of the infection.

Materials and methods

Samples

The samples included in the present study were sagittal sections of cerebellum from 18 classical Scrapie sheep (ARQ/ARQ in all cases with the exception of one ARR/ARQ) and four atypical Scrapie sheep (ARR/ARR, ARQ/ARQ141L/F, ARR/ARQ) from various flocks of the region of Aragón and three atypical Scrapie sheep (ARR/ARQ, AF141RQ/AF141RQ, AF141RQ/AHQ) from Cataluña. Samples corresponding to negative animals from flocks in which Scrapie cases had never been described were used as negative controls. The studies developed were approved by the Ethical Committee for Animal Welfare from the University of Zaragoza.

Immunohistochemistry

Serial 50-μm sections were pre-treated for epitope retrieval as follows (Monleón et al. 2004): immersion in 98 % formic acid for 15 min, proteinase K treatment for 15 min at 37 °C and heat treatment. This last step was slightly modified in this study, substituting autoclaving (15 min) for 80 °C (overnight). The sections were then incubated with the respective monoclonal prion-specific antibody and polyclonal anti-GFAP (1/500; DAKO, Hamburg, Germany) overnight with agitation at 4ºC. Subsequently, streptavidin-conjugated Alexa 594 (1/200; Invitrogen, Eugene, Ore., USA) and Alexa 488 (1/200; Invitrogen) were used as secondary antibodies for 1 h in darkness. Incubation for 1 h with IgG anti-mouse biotin (1/100; Invitrogen) was performed prior to fluorochrome addition in order to enhance the fluorescent signal for prion proteins. L42 (1/250; Bio-Pharm, Darmstadt, Germany), P4 (1/160; Bio-Pharm) and R145 (1/500; DEFRA, UK) were all tested as primary antibodies for visualizing PrPsc deposits in order to ensure that the results did not depend on the antibody used.

The same protocol was applied to samples with neither primary nor secondary antibodies with the aim of controlling for autofluorescence presented by the tissue. Additionally, the same immunohistochemical protocol, except for using EnVision (DAKO, 30 min at room temperature) and diaminobenzidine (DAKO, Hamburg, Germany) as the visualization system and chromogen, respectively, was carried out with the aim of comparing the results provided by confocal and light (conventional immunohistochemistry) microscopy.

Confocal microscopy

Each section was assessed by confocal imaging. Serial confocal Z-stacks were taken on a Zeiss laser-scanning confocal microscope LSM 510 (Carl Zeiss MicroImaging, Germany) with 10× (NA 0.3) and 20× (NA 0.5) objectives.

Fluorescence emission resulted from excitation with a 488-nm and a 594-nm laser and was detected with a two-channel multi-track configuration, by using band-pass 505- to 530-nm and long-pass 615-nm filters, respectively. Z-stacks were finally combined in one stack for each sample and processed by Zen 2008 software (Carl Zeiss MicroImaging).

Results

The high background obtained in the images seriously complicated the set-up of the applied technique. Because the fixation times and the subsequent storage of the tissues were too long, the antibody penetration was hampered and therefore, retrieval procedures were required in order to obtain the optimal immunofluorescence (Shi et al. 1991). In order to establish the protocol presented in the Materials and methods, several modifications were necessary to reduce the autofluorescence and diminish the background. The high autofluorescence was probably caused by, in addition to the storage conditions mentioned above, lipofuscin granules, which have been demonstrated to be normally present throughout the brain (Romijn et al. 1999; Kovacs et al. 2002). In order to reduce background, the same sections were screened by excitation with several lasers to determine in which spectral band width an absence or a decrease of autofluorescence could be detected.

Furthermore, because of the pre-treatment aimed at unmasking the epitopes and because all the sections were acquired independently, difficulties were experinenced in precisely illustrating the same areas within the section for the various antibodies used. Additionally, imaging showed intensity variations within each section when they were newly immunolabelled with several antibodies, because of the independent acquisition of the images (Ermolayev et al. 2009).

Negative samples showed no PrPsc deposits, regardless of the primary antibody tested (Fig. 1). However, all positive samples showed PrPsc immunostaining with all primary antibodies tested. Immunolabelling was seen over the granular and molecular layers, presenting the same granular-spot type deposition in all classical (Fig. 2) and atypical (Fig. 3) samples included in the study.

The presence of PrPsc in Purkinje cells was not observed in any sample included in the study or with any of the primary antibodies tested, even in those eight cases in which PrPsc appeared to be accumulated in these cells according to conventional immunohistochemistry (Fig. 4). A proposal that the cells had been damaged by the pre-treatment applied to the samples and were unable to be visualized was discarded, since the same protocol was undertaken for conventional optical microscopy and the cells could be clearly stained.

Concerning the co-localization studies, prion protein and glial cells co-localized in several areas. Specifically, three cases of co-location occurred in the Bergmann glia, although this was mainly seen in astrocytes corresponding to the marginal glia (Figs. 2, 3, yellow). This co-localization was found in all positive studied cases, to a greater or lesser extent, regardless of the prion-specific antibody applied and with no differences between classical and atypical samples.

Finally, the comparative study performed between samples from classical and atypical Scrapie showed no differences regarding co-localization in the studied region of the cerebellum when using L42 as the primary antibody. Moreover, no differences were seen when primary antibody was replaced by P4 or R145 (Figs. 2, 3).

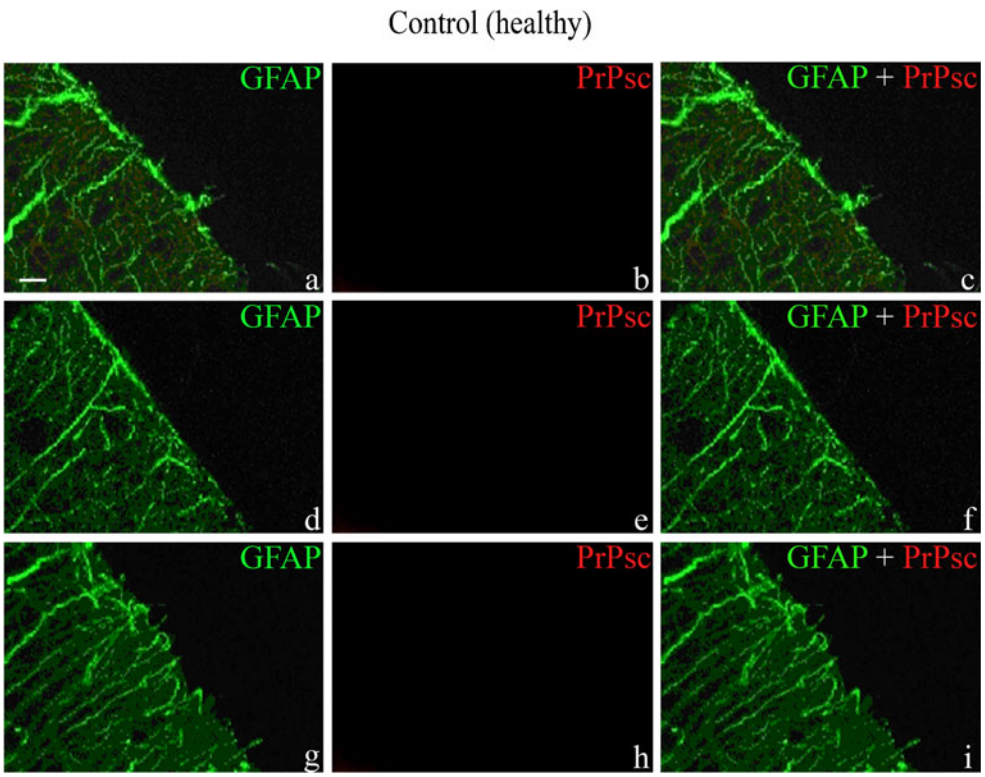

Fig. 1 Images obtained by confocal microscopy corresponding to no co-localization (no yellow colour) between PrPsc and marginal glia in control healthy animals as revealed by using L42 (**a–c**), P4 (**d–f**) or R145 (**g–i**) and anti-GFAP as primary antibodies. *Bar* 10 μm

Discussion

PrPsc was distributed as fine granules, as observed in CJD (Kretzschmar et al. 1991) and it was similar to that described by conventional immunohistochemistry in the granular and molecular layer (Armstrong et al. 2009). The connection of PrPsc to astrocytes was detected, as previously described (Jeffrey et al. 2004), with localization in and

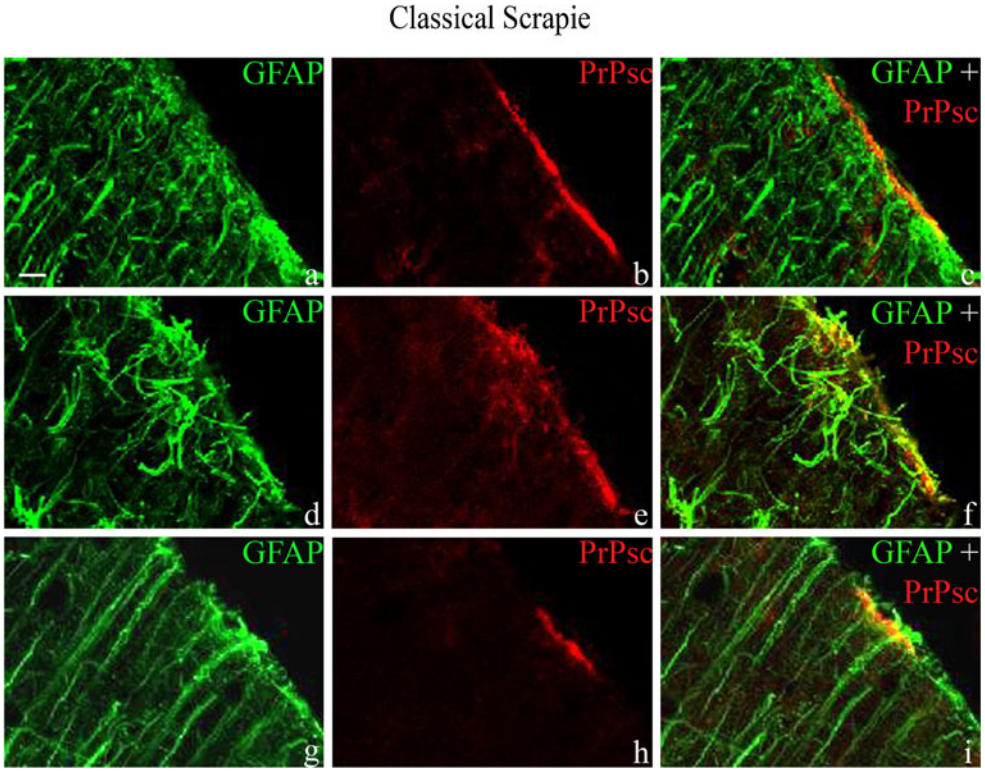

Fig. 2 Images obtained by confocal microscopy corresponding to co-localization (*yellow*) between PrPsc and marginal glia in classical Scrapie by using L42 (**a–c**), P4 (**d–f**) or R145 (**g–i**) and anti-GFAP as primary antibodies. *Bar* 10 μm

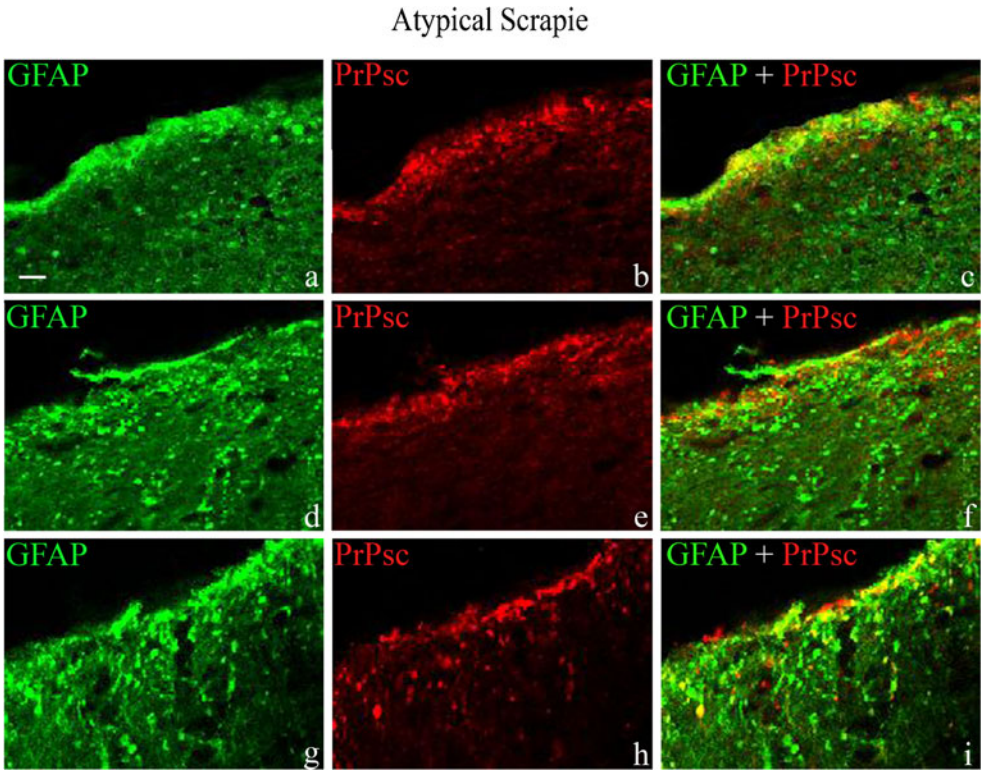

Fig. 3 Images obtained by confocal microscopy corresponding to co-localization (*yellow*) between PrPsc and marginal glia in atypical Scrapie by using L42 (**a–c**), P4 (**d–f**) or R145 (**g–i**) and anti-GFAP as primary antibodies. *Bar* 10 μm

around hypertrophic GFAP-positive astrocytes. On the other hand, co-localization in the cerebellum, which was initially investigated because the deposit is more evident relative to other areas in atypical Scrapie, was supported in the present study because a notable presence of PrPsc in both layers and in both classical and atypical samples has been detected, regardless of the primary antibody applied (Benestad et al. 2003; Onnasch et al. 2004; Nentwig et al. 2007; Moore et al. 2008).

The absence of PrPsc in Purkinje cells is probably one of the most relevant findings provided in the present study, because, in some cases, PrPsc deposits seem to be located in Purkinje cells according to conventional immunohistochemistry (van Keulen et al. 1995; Ye et al. 1998b). Harris et al. (1993) have found PrP in high concentrations in Purkinje cells and some studies have shown a high level of PrP mRNA in these cells, suggesting the involvement not only of PrPc (Manson et al. 1992) but also of PrPsc (Kretzschmar et al. 1986). However, in accordance with the results provided here, PrPsc seems to have an exclusively extracellular location. This finding agrees with other studies that have been unable to find detectable levels in Purkinje cells (Kretzschmar et al. 1991; Moore et al. 2008), even in atypical cases (Onnasch et al. 2004). The present study demonstrates that this technique constitutes a suitable tool for revealing the relative location of the protein of interest, offering a higher resolution because it provides results in the z-plane (Romijn et al. 1999). This improved resolution allowed by the confocal protocol thus establishes this imaging technique as a method of choice for developing such co-localization studies.

Results obtained by using conventional immunohistochemistry have suggested the co-localization of prion protein

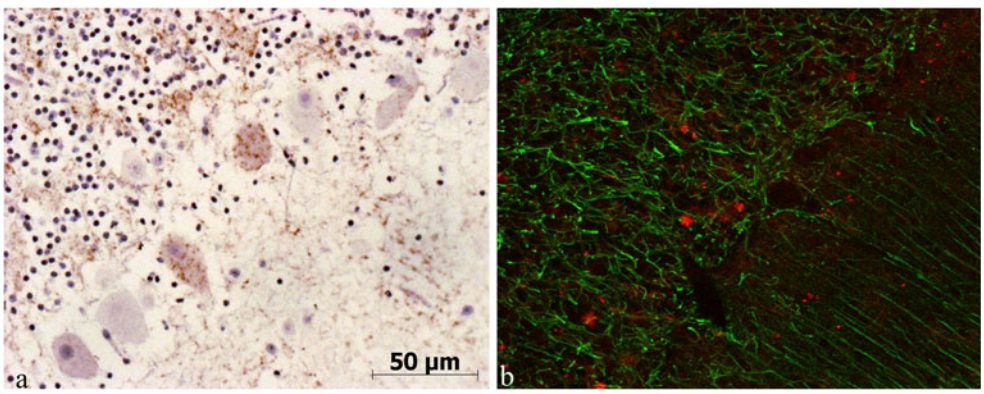

Fig. 4 Location of PrPsc deposits in relation to Purkinje cells, shown in **a** by conventional immunohistochemical and in **b** by confocal techniques (exclusively extracellular)

and glial cells (Ye et al. 1998b; Andréoletti et al. 2002). Specifically, co-localization with astrocytes has been found inside the lisosomes (Jeffrey et al. 2004). In the Bergmann glia, studied by Yamada and Watanabe (2002), immunoreactive GFAP has been described in the fibres coursing through the molecular layer of the cerebellum and in astrocyte fibres surrounding vacuoles in the granular layer (Diedrich et al. 1991), appearing similar to the Bergmann gliosis seen in cases of CJD (Ferrer et al. 2000). Although a high proportion of PrPsc in the molecular layer has been observed in Bergmann radial glia and their processes (Safar et al. 2005), nothing has been said conclusively about the possible co-localization with PrPsc. In this study, PrPsc co-localization with astrocytes of the marginal glia has been observed in all cases. This finding confirms the co-localization in limitans glia as seen by van Keulen et al. (1995) in other regions of the cerebrum and supported by the increase of GFAP mRNA found by Kretzschmar et al. (1986). Co-localization has also been found by using immunogold detection with electron microscopy to examine the region around the limitans glia of perivascular astrocytes or astrocytes in the sub-pia (Jeffrey et al. 2004). Deposits seen along the marginal glia appear similar to the accumulation of PrPsc in the pia mater, in the outer limitans glia, found by van Keulen et al. (1995).

In the case of atypical samples, the evidence of this co-localization is novel and even contradicts one of the most relevant findings of the study of Moore et al. (2008) suggesting that the agent has no, or very low, tropism for glial cells, because the PrPsc immunostaining associated with glial cells is seen only in classical cases and not in the atypical ones. The presence of co-localization in both the classical and the atypical samples suggests that the role of astrocytes with regard to the prion proteins is the same in both pathologies.

These findings indicate the involvement of glial cells in prion propagation, a much-discussed question in studies in which axons (Taraboulos et al. 1992) or direct neuroanatomical interconnections (Hecker et al. 1992) have been seen to influence prion transport. On the other hand, some protoplasmatic astrocytes are known to send processes to neighbouring blood vessels in which these processes form endfeet plastering the capillary wall and participating in the formation of the blood-brain barrier (Rodríguez et al. 2009).

The co-localization of PrPsc with the marginal glia suggests the haematogenous way as a possible means for prion propagation. This assumption was suggested when Sisó et al. (2009) described the involvement of the circumventricular organs in prion propagation or when Pattison and Jones (1967) found hypertrophy of astrocytic end-plates near blood vessels in their study. Furthermore, several other authors have demonstrated deposits that are located around blood vessels (Miller et al. 1993; Ryder et al. 2001) and that probably contribute to the perivascular limitans glia (van Keulen et al. 1995). In this sense, further studies are needed in order to determine whether prion proteins can be interchanged in the same manner as other metabolic products that pass across the endothelial cells of blood vessels before accessing central nervous system tissue.

The real role of astrocytes remains under debate. Despite studies suggesting neuronal PrPc as a necessary requirement for in vitro toxicity (Brown et al. 1994) and studies supporting the presence of PrPd in astrocytes while not describing an ultrastructurally specific pathology of TSE (Jeffrey et al. 2004), the necessary involvement of astrocytes in the development of Scrapie has been confirmed in the present study. Additionally, PrPsc has been shown not to be toxic to neuronal culture without PrPc, unless they are cultured with astrocytes (Brown 1999). Moreover, mice only expressing PrPc in their astrocytes can succumb to Scrapie (Raeber et al. 1997) and can show its typical pathology, including vacuolization and gliosis (Jeffrey et al. 2004). Therefore, the present study has demonstrated the relevance of astrocytes in the pathology of the disease.

In conclusion, this study demonstrates the usefulness of co-localization studies in helping to reveal the possible specific pathological potential of astrocytes both in TSEs and in various types of dementias in which the pivotal role of astroglia is widely accepted.

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