Subcellular Localization of Disease-Associated Prion Protein in the Human Brain

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Disease-associated prion protein (PrP^{TSE}) deposits in distinct immunostaining patterns in the brain in Creutzfeldt-Jakob disease, including synaptic, extracellular, and cell-associated localizations. After having developed an appropriate pretreatment protocol to enhance immunostaining for PrP^{TSE} without damaging epitopes of other antigens, we systematically evaluated co-localization patterns of distinct PrP^{TSE} immunodeposits by confocal laser microscopy, including optical serial sectioning. As shown by quantification, the most prominent co-localization of PrP^{TSE} is with synaptophysin, but PrP^{TSE} may also co-deposit with connexin-32, a gap junction-related protein. Furthermore, neuronal cell bodies, dendrites, axons, astrocytes, and microglia harbor granular PrP^{TSE} deposits. Highly aggregated deposits are focally ubiquitinated. We conclude that PrP^{TSE} is not exclusively associated with chemical but also with electric synapses, axonal transport may be a relevant route of PrP^{TSE} spread in the brain, and activated microglia and astrocytes may play a role in PrP^{TSE} processing, degradation, or removal. (*Am J Pathol* 2005, 166:287–294)

Post-translational conversion of the α -helix-dominant cellular prion protein (PrP^C) into the β -sheet-dominant PrP^{Sc} is a common characteristic of animal and human prion diseases including Creutzfeldt-Jakob disease (CJD).¹ Both protease-resistant and protease-sensitive forms of PrP^{Sc} are known.² Due to the lack of unequivocally conformation-specific antibodies, immunohistochemical differentiation of PrP^C from PrP^{Sc} has remained a matter of debate and creates a barrier for the evaluation of disease process. However, detailed immunohistochemical de-

scriptions define patterns which are specific for disease,³ thus the term disease-associated PrP (labeled PrP^{TSE} in the present study) is suitable to label conformationally uncharacterized deposits of PrP observed only in prion-diseased brains by immunohistochemistry.

In the diseased human brain, main deposition patterns of PrP^{TSE} are so-called diffuse/synaptic, patchy/perivacuolar, perineuronal, and plaque-like patterns.³ The first is characterized by a diffuse fine-granular distribution of anti-PrP^{TSE} immunoreactivity resembling the distribution of the presynaptic protein synaptophysin (hence the term "synaptic pattern"). Presynaptic domains appear to be the privileged site of PrP^C, as has been confirmed by double labeling with synaptophysin.⁴ Also a post-synaptic localization of PrP^C is sometimes observed in synapses of the central nervous system (CNS) and muscle-nerve synapses.⁴ Others suggest that PrP^C is almost excluded from synaptic vesicles and also describe its presence in the cytosol of certain neuronal subpopulations.⁵ Cell culture studies indicated a destiny of PrP^C for the plasma membrane and caveolae-like membranous domains.⁶ Immunogold electron microscopy has demonstrated presence of PrP epitopes in a presynaptic localization in human variant CJD and bovine spongiform encephalopathy-infected monkey brain.^{4,7} Additionally, subcellular fractionation has shown that the highest concentration of PrP^{TSE} in CJD brain is found in the synaptosomal fraction, however, without distinguishing between pre- and post-synaptic localization.⁸

Experimental data suggest that PrP^{TSE} may also be found in other parts of neurons than their synaptic terminals, furthermore, there is also evidence for accumulation of PrP^{TSE} in astrocytes and microglia.⁴ Due to methodological difficulties, confirmatory evaluation of PrP^{TSE}

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Table 1. Summary of Antibodies Used in This Study

Antibody	Supplier	Dilution	Clonality	Specificity (labeled structures)
12F10	Cayman Chemicals, Ann Arbor, MI	1:300	Mono (Mouse) IgG2a	PrP
SMI-31	Sternberger Mo. Ltd., Lutherville, MD	1:2500	Mono (Mouse) IgG1	Phosphorylated epitope in extensively phosphorylated neurofilament H and, to a lesser extent, with neurofilament M. Reacts broadly with thick and thin axons and some dendrites such as basket cell dendrites, but not Purkinje cell dendrites. Nerve cell bodies are generally unreactive.
SMI-32	Sternberger Mo. Ltd., Lutherville, MD	1:100	Mono (Mouse) IgG1	Non-phosphorylated epitopes in neurofilament H. Reacts with neuronal cell bodies and dendrites and only some thick axons.
AP20	Chemicon, Temecula, CA	1:250t	Mono (Mouse) IgG1	Microtubule associated protein-2 (dendrites + neuronal body and occasional axons)
SY38	Dako, Glostrup, Denmark	1:10	Mono (Mouse) IgG1	Synaptophysin (presynaptic structures, synaptic vesicle)
Anti-synapsin I	Alexis Biochemicals, Grünberg, Germany	1:25	Poly (Rabbit)	Synapsin I (synaptic vesicles)
M12.13	Zymed, San Francisco, CA	1:25	Mono (Mouse) IgG	Connexin-32 (gap junction protein-electrical synapses)
Anti-GFAP	Dako, Glostrup, Denmark	1:2000	Poly (Rabbit)	Glial fibrillar acidic protein (reactive astroglia)
CR3/43	Dako, Glostrup, Denmark	1:50	Mono (Mouse) IgG1	HLA-DR (reactive microglia)
Anti-ubiquitin	Dako, Glostrup, Denmark	1:100	Poly (Rabbit)	Ubiquitin (ubiquitinated protein aggregates)

deposition in the diseased human brain is lacking. After having developed an appropriate pretreatment protocol to visualize immunostaining for PrP^{TSE} without damaging epitopes of other antigens, we systematically studied PrP^{TSE} deposition by double immunofluorescent labeling and evaluated co-localization using laser confocal microscopy.

Materials and Methods

Four autopsy cases of sporadic Creutzfeldt-Jakob disease (sCJD), with full-length analysis of the prion protein gene (*PRNP*), were selected from the tissue bank of the Institute of Neurology, Medical University Vienna, Austria. We selected CJD cases with relatively preserved cortical structure along with high amounts of immunohistochemically detectable PrP^{TSE}. Cases 1 (72-year-old female, 5-month disease duration) and 2 (65-year-old female, 1-month disease duration) were methionine/methionine (MM) homozygotes at the polymorphic codon 129 of *PRNP*; case 3 (74-year-old male, 6-week disease duration) was a VV homozygote; and case 4 (67-year-old female, 12-month disease duration) was a methionine/valine (MV) heterozygote. Two control cases without prion disease were included as well (control case 1: 78-year-old male, cause of death, myocardiac insufficiency; control case 2: 78-year-old female, cause of death, myocardiac infarction). In all cases, brain tissue samples containing cerebral cortex and white matter were routinely fixed in 4% buffered formalin. This was performed within 12 hours (CJD cases 2 and 3), 24 hours

(CJD case 4 and control case 1), 48 hours (CJD case 1 and control case 2) after death. Tissues were stored in formalin for 5 days (CJD case 1), 5 weeks (CJD case 2), 2 months (CJD case 3), 6 days (CJD case 4), 11 days (control case 1), and 4 days (control case 2) before paraffin-embedding. Paraffin-embedded tissue blocks were stored in darkness and at room temperature for 36 months (CJD case 1), 1 month (CJD case 2), 18 months (CJD case 3), 1 month (CJD case 4), 2 months (control case 1), and 2 weeks (control case 2) before being processed for this study as specified below.

Immunohistochemistry and Laser Confocal Microscopy

Five- μ m thick sections were obtained from each tissue block for conventional immunohistochemistry and double immunofluorescent labeling. Twenty- μ m thick tissue sections were used for optical serial sectioning ("z-stack-ing"). After a pilot fashion testing of different pretreatment protocols suitable to detect both PrP^{TSE} and selected antigens, we defined a protocol that included 20 minutes of autoclaving (100°C) in citrate buffer (pH 6.0) and incubation for 2 minutes in formic acid (96%).

Commercially available primary antibodies with well-defined specificity used for this study are listed in Table 1. We used the monoclonal anti-PrP antibody 12F10 that was previously shown to be the most reliable in detecting disease-associated PrP.³ Sections were incubated overnight at room temperature with each antibody.

Figure 1. a–l: The modified pretreatment protocol allows satisfactory immunohistochemical detection of diffuse/synaptic (a–c), patchy/perivacuolar (b), perineuronal (c) PrP^{TSE} deposits in sporadic, codon 129 MM (a–b), and VV (c) homozygote CJD cases. Plaque-type PrP^{TSE} deposits are observed in a codon 129 MV (d) CJD case (magnification, $\times 10$, a–d, left; magnification, $\times 40$, a–d, right). In addition, synaptophysin (e), connexin-32 (f), phosphorylated neurofilaments (g), unphosphorylated neurofilaments (h), MAP-2 (i), HLA-DR (j), GFAP (k), and ubiquitin (l) are satisfactorily immunolabeled using the same pretreatment protocol (all case 3 except h which is case 4; magnification, $\times 40$). **m–t:** The standard three-tiered pretreatment protocol for PrP^{TSE} allows immunohistochemical detection of synaptic (m) and perivacuolar/patchy (n) PrP^{TSE} deposits using antibody 12F10 but strongly reduces or abolishes immunoreactivity against synaptophysin (o), connexin-32 (p), phosphorylated neurofilaments (q), unphosphorylated neurofilaments (r), MAP-2 (s), and HLA-DR (t) (m and o–t show case 3, n shows case 2; magnification, $\times 40$).

For double immunolabeling, the fluorescent-labeled secondary antibody for the anti-PrP antibody 12F10 was Alexa Fluor 488 goat anti-mouse IgG (1:200, Molecular Probes, USA), for anti-SMI-31, anti-SMI-32, anti-MAP-2, anti-connexin-32, anti-synaptophysin, and anti-HLA-DR Alexa Fluor 546 goat anti-mouse IgG (1:200, Molecular Probes), and for synapsin, GFAP, and ubiquitin goat anti-rabbit IgG (1:200, Molecular Probes). Primary antibodies were visualized with fluorophore-labeled, anti-mouse Fc, Fab fragments, using the Zenon Alexa Fluor 488 Mouse IgG_{2a} Labeling Kit (Molecular Probes, Leiden, The Netherlands) for 12F10, and the Zenon Alexa Fluor 546 Mouse IgG₁ Labeling Kit (Molecular Probes, Leiden, The Netherlands) for other antibodies. We used argon 488-nm and helium/neon 633-nm and 543-nm lasers to elicit immunofluorescent staining. Immunofluorescence labeling was evaluated using a Zeiss LSM 510 laser scanning confocal microscope. The optical pinhole was adjusted for each scanning experiment to obtain optical slices with a 0.2- μm thickness. Optical serial sections with distances of 0.5 μm between individual sections were obtained to confirm intracellular localization of PrP^{TSE} deposits. To compare the proportion of co-localized pixels of PrP^{TSE}/synaptophysin and PrP^{TSE}/connexin-32 we extracted 10 randomly selected 1000 μm^2 areas for quantification from the scanned images of double immunolabeling. Accepting bright yellow color as a marker of co-labeling of green and red particles, we used Adobe Photoshop 6.0 to select one bright yellow-colored pixelbox from each of the saved images. Pixelboxes containing bright yellow components were highlighted and counted manually on the screen. A similar procedure was performed for green color. We then calculated the per-

centage of pixelboxes containing a yellow component from the total number (yellow+green) of counted pixelboxes.

Results

By testing pretreatment protocols, we have defined a protocol suitable for our study. This enabled us to detect either diffuse/synaptic, patchy/perivacuolar, perineuronal, or plaque-type PrP^{TSE} deposits sufficiently after this pretreatment (Figure 1, a to d). In comparison with our standard immunohistochemical pretreatment protocol,³ the signal was slightly less intense but labeled all structures similarly (Figure 1, m and n). In addition, all other antibodies used in our study convincingly detected their respective antigens with this protocol (Figure 1, e to l) in contrast to the loss of immunoreactivity after using the three-tiered pretreatment protocol for PrP^{TSE} (Figure 1, o to t). In normal control brains we found no immunoreactivity for PrP^{TSE}, and we did not observe immunolabeling of any cells in the CNS (Figure 4).

PrP^{TSE} and Synapses: Chemical and Electric

In all cases, fine granular, so-called diffuse/synaptic type, of PrP^{TSE} deposits co-localized with synaptophysin (Figure 2a). A similar co-labeling pattern was seen with synapsin I (data not shown). Quantification analysis revealed co-localization of PrP^{TSE} with synaptophysin in $72.91 \pm 5.9\%$ of pixels. Coarse PrP^{TSE} deposits like patchy/perivacuolar or plaque type did not show co-localization with

Figure 2. Confocal laser scanning microscopy of double immunofluorescent labeling for PrP^{TSE} (a–g; green) and synaptophysin (a), connexin-32 (b), phosphorylated neurofilaments (c–e), unphosphorylated neurofilaments (f), and MAP-2 (g) using an appropriate pretreatment protocol. Presynaptic structures (a), gap junction-related components (b), axons (c–e), neurons and neuronal processes (f–g) are labeled with red. Autofluorescence (eg, lipofuscin) is labeled with blue. Co-localization is shown by yellow color. Optical sections are 0.2-μm thick. **c:** Split image showing PrP^{TSE} granules following the outline of an axon, thus indicating intra-axonal localization of PrP^{TSE} granules. Intra-axonal localization of PrP^{TSE} granules is confirmed by optical serial sections (individual sections are 0.2-μm thick and 0.5 μm apart) showing that individual PrP^{TSE} granules are surrounded by axoplasm on all sides (d–e). The area highlighted in (d) by a **white box** is shown in (e). Further, we observed clustering of granular PrP^{TSE} immunoreactivity within the perikaryon in close association with the surface of neurons (f) and within dendrites (g). Additionally there is perineuronal and periaxonal deposition of PrP^{TSE} (f). On the **right** of (a), (b), (f), and (g), **pixelboxes** of three selected areas showing co-localization are enlarged. Fine granular (diffuse/synaptic) PrP immunodeposits are seen in all pictures.

synaptophysin. Co-labeling of fine granular PrP^{TSE} and connexin-32 was shown in 10.78 ± 3.8% of pixels (Figure 2b).

PrP^{TSE} and Neurons: Processes and Cell Body

Using the SMI-31 antibody to immunolabel axons, we noted co-localization with PrP^{TSE} within the axon (Figure 2, c to e). This was also evident after creating split images where the incorporated PrP^{TSE} granular deposits follow the outline of an axon (Figure 2c). Intra-axonal localization of PrP^{TSE} granules was confirmed by optical serial sections showing that individual PrP^{TSE} granules are surrounded by axoplasm on all sides (Figure 2, d and e). Infrequently, we observed clustering of granular PrP^{TSE} immunoreactivity within the perikaryon in close association with the surface of neurons (Figure 2f) and dendrites (Figure 2g) of neurons. Additionally there is perineuronal and periaxonal deposition of PrP^{TSE} (Figure 2f). None of the neuronal markers showed co-labeling with PrP^{TSE} in aggregated patchy/perivacuolar deposits.

PrP^{TSE} and Glia: Astro- and Microgliosis

Rarely, we observed PrP^{TSE} deposits within micro- (Figure 3, a and b) and astroglial (Figure 3, c to e) cells. Similarly to the intra-axonal distribution, intracytoplasmic astro- and microglial localization of PrP^{TSE} deposits was confirmed by optical serial sectioning, showing that PrP^{TSE} deposits are surrounded by cytoplasm on all sides. Occasionally PrP^{TSE} deposits were clustered in close vicinity of astroglial cells that also showed intracytoplasmic accumulation of PrP^{TSE} (Figure 3e).

Ubiquitination of PrP^{TSE}

Parts of extensive aggregated deposits of PrP^{TSE} co-localized with ubiquitin frequently (Figure 2f) while fine granular deposits do not appear to be ubiquitinated.

Discussion

Immunohistochemistry for PrP is a powerful tool for diagnosing prion disease.⁹ To avoid misinterpretation caused by the inability of available antibodies to distinguish between normal and pathological conformers of PrP, tissues must undergo special pretreatment before immunostaining to abolish PrP^C and to enhance PrP^{TSE} immunoreactivities. Commonly used pretreatment protocols use the combination of hydrated or hydrolytic autoclaving at 121°C of formic acid, guanidine thiocyanate, and proteinase K.³ However, these pretreatment protocols also damage other antigens present in the tissue, making their immunodetection impossible. This is one reason for the paucity of co-labeling studies defining the exact localization of PrP^{TSE} in the CNS.

Regional distribution of PrP^{TSE} was shown to be different in sporadic, iatrogenic, and genetic prion disease cases.¹⁰ Later molecular classification based on Western blot characteristics of the protease-resistant PrP and the

PRNP polymorphism at codon 129 has revealed that the PrP immunostaining patterns are distinguishable in anatomical regions and molecular phenotypes of sporadic CJD cases.^{11,12} In our present study we collected samples harboring all major morphological subtypes of PrP^{TSE} immunodeposits related to sporadic CJD brains.

Co-localization of PrP^{TSE} deposits with synaptophysin and synapsin I confirms previous ultrastructural studies that PrP^{TSE} is present in presynaptic components. Quantification analysis, however, suggests the existence of other sites of PrP^{TSE} deposition. Surprisingly, we observed co-labeling of fine granular PrP^{TSE} and connexin-32. Connexins are molecular constituents of gap junctions (electric synapses) that are distributed in astrocytes, oligodendrocytes, and neurons.^{13,14} Connexin-32 is predominantly related to neurons, less to oligo-, and not to astrocytes.¹⁴ Interestingly, mainly GABAergic interneurons are interconnected through electric synapses, a subset of which is particularly vulnerable in human and experimental prion diseases.^{15,16} Accumulation of PrP^{TSE} in gap junctions may be a substrate for neuronal damage as the role of these structures involves intra- and extracellular homeostasis, trafficking and supply of energy-producing substrates to neurons, or neuroprotection.¹⁴

We observed unequivocal localization of PrP^{TSE} granules within axons. Previous ultrastructural studies have suggested association of PrP^{TSE} with the plasmalemma of neurites,^{17,18} however, PrP^{TSE} has never been detected inside neurites. Presence of PrP^{TSE} in the cytosol of axons is not unexpected, since a recent study demonstrated the existence of cytosolic PrP^C probably following a retro-translocation from the endoplasmic reticulum.⁵ As PrP^{TSE} has been shown to traffic also in endosome-like organelles,¹⁹ it is also conceivable that our demonstration of intra-axonal PrP^{TSE} has such sub-cellular basis. However, our observation merits (immuno)-ultrastructural confirmation to clarify whether PrP^{TSE} within the axons is free in the cytosol or related to vesicles (eg, endosomal). Recent observations in experimental models suggest that transport of PrP^{TSE} along peripheral nerves to the CNS is following a domino-like manner along PrP^C-expressing nerve membranes rather than being achieved via conventional axonal transport.²⁰ In human prion disease PrP^{TSE} deposits in the CNS may be observed in both fine and coarse perineuronal patterns supporting a domino-like manner of propagation. However, in human disease the perineuronal pattern is not predominant. It is seen mainly in VV or MV type 2 subtypes of sporadic CJD cases and variant CJD, suggesting that this might be a strain-dependent phenomenon.^{3,21,22} In contrast, fine granular, so-called diffuse/synaptic, deposits of PrP^{TSE} represent the major morphological appearance. We thus suggest that PrP^{TSE} in the CNS follows at least two pathways, one along and one within neurites. Although PrP^C is subject to fast anterograde transport,²³ other possibilities need to be elucidated. One important route is probably ad-axonal, however, in circumstances with overwhelming production of PrP^{TSE}, which is more likely to happen in the CNS and not

Figure 3. Confocal laser scanning microscopy of double immunofluorescent labeling with 12F10 (**a–f**, green) and HLA-DR (**a–b**), GFAP (**c–e**), and ubiquitin (**f**) using an appropriate pretreatment protocol. Microglia (**a–b**), astrocytes (**c–e**), and ubiquitin (**f**) are labeled with red. Autofluorescence is demonstrated by blue color in the **right** lower corner **inset** (**e**). Optical sections are 0.2- μ m thick. We observed granular PrP^{TSE} deposits in the cytoplasm of microglial cells (**a**) and astrocytes (**c–e**). Periastrcytic accentuation of PrP^{TSE} deposits is noted (**c**). On the **right** of (**a–f**), **pixelboxes** of three selected areas are enlarged. Fine granular (diffuse/synaptic) PrP immunodeposits are seen in (**a** and **c**) (case 1), (**e**) (case 3), while coarse patchy/perivacuolar immunodeposits are seen in (**b**, **d**, and **f**) (case 2). Magnification, $\times 100$ (**a–f**).

in the periphery, conventional axonal transport systems may participate in distribution of PrP^{TSE}.

Clusters of granular PrP^{TSE} immunoreactivity within the perikaryon in close association with the surface of neurons and dendrites of neurons must be distinguished

from an occasional diffuse cytoplasmic immunolabeling of neurons which has also been described in non-diseased brains and is considered to represent cellular PrP.^{24,25} The anti-PrP antibody (12F10) applied in our present study does not show this type of diffuse neuronal

Figure 4. Confocal laser scanning microscopy of double immunofluorescent labeling with 12F10 (**a–d**) and anti-GFAP (**a**), SMI 31 (**b**) anti-synaptophysin (**c**), and anti-connexin-32 using the modified pretreatment protocol in a non-CJD brain (control case 1; blue signals indicate autofluorescence, eg, lipopigment). No green PrP deposits are detectable, while astrocytes (**a**), axons (**b**), presynaptic structures (**c**), and gap junction-related components (**d**) are immunolabeled (red).

labeling.³ The granular deposits in the cell body and dendrites suggest a post-synaptic localization of PrP^{TSE}. This is compatible with previous descriptions of a post-synaptic localization of cellular PrP,⁴ which is considered to be a prerequisite for formation of PrP^{TSE}. We confirm previous ultrastructural studies that have already noted PrP^{TSE} in neuronal perikarya that was proposed to be mainly related to the lysosomal-endosomal system there.⁴ The PrP^C-PrP^{Sc} conversion was suggested to use the endocytic pathway via the caveola-like domain membrane or coated pits to the endosomal-lysosomal system. The latter were suggested to be key organelles in the processing route toward production of PrP^{Sc} not only in cell culture, but also in animal TSE and human variant CJD brains.^{7,26} Having found no neuronal markers within aggregated patchy/perivacuolar or plaque-like deposits we excluded the possibility that neuronal debris is a component of these amorphous materials.

Our observations that micro- and astroglial cells harbor intracytoplasmic PrP^{TSE} granules and that there is periastrorcytic accumulation of PrP^{TSE} deposits suggest that both microglial and astroglial cells may have a role in the processing, degradation, or removal of PrP^{TSE}. Whether microglia are capable of phagocytosing aggregated PrP^{TSE} material has rarely been addressed in research, although prominent microgliosis is a characteristic finding in CJD brains. Only a recent study demonstrated that spleen macrophages actively participate in the clearance of intraperitoneal scrapie inoculum.²⁷ Activation of micro- and astroglial cells at sites of aggregated PrP^{TSE} deposits and accumulation of PrP^{TSE} in lysosomes have been documented previously.²⁸ In an *in vitro* model using prion fragment 106–126 it has been shown that microglia is

needed for neurotoxicity, while the same fragment stimulates proliferation of astrocytes.^{29,30} Microglia activation occurs early in disease but there is neither direct *in vivo* evidence that PrP^{Sc} is proinflammatory nor was it demonstrated to what microglial cells actually respond.²⁹

Ubiquitination of PrP^{TSE} was already suggested by demonstrating ubiquitin immunoreactivity in areas where PrP^{TSE} deposits are observed (eg, as perivacuolar immunopositivity).³¹ Here we confirm that highly aggregated PrP^{TSE} deposits indeed co-localize with ubiquitin frequently. That disease-associated PrP may be ubiquitinated is supported by a recent study, using immunoprecipitation and sandwich ELISA, showing ubiquitination of PrP after developing protease resistance in the brains of scrapie-infected mice.³²

In sum, our study suggests alteration of both chemical, indicated by the presynaptic protein synaptophysin, and electric synapses, indicated by the gap junction-related protein connexin-32, by accumulated PrP^{TSE}. This might be a central substrate of prion disease pathogenesis and clinical deterioration either by abolishing homeostatic communication between cells or by releasing inhibition. By demonstrating PrP^{TSE} in the neuronal cell body and dendrites, a role of post-synaptic, in addition to previously assumed presynaptic, structures in CJD pathogenesis is proposed. The observation of both intra- and ad-axonal localization of PrP^{TSE} suggests that both intra-axonal transport as well as domino-like propagation along the axonal surface may occur in the CNS. Moreover, our results suggest that PrP^{TSE} may be processed or degraded in micro- or astroglial cells.

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