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Transmissible mink encephalopathy – review of the etiology of a rare prion disease

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Abstract

We review here the history, neuropathology, clinical picture and molecular data on transmissible mink encephalopathy (TME). This obscure disease is of utmost importance as it is plausible that it represents a transmission of hidden bovine spongiform encephalopathy (BSE) to mink in the USA. Of special interest is the similarity of L-type of BSE and TME. Furthermore, experimental molecular studies showed the TME strain-specific in vitro conversion in a cell-free system. In addition, we show here for the first time confocal laser microscopy studies of co-localization of PrP^d- amyloid plaques and GFAP-expressing astrocytes.

Key words: *prion, transmissible mink encephalopathy, TME, confocal laser microscopy.*

History of TME

Transmissible mink encephalopathy (TME) is a rare disease of ranch-reared mink (*Mustela vision*) (Fig. 1) described in 1965 by Hartsough and Burger [15,28]. The first outbreak was noticed in 1947 in a farm in Brown County, Wisconsin, USA and fatality reached almost 100% of the adult mink. Insight into the nature of infection was evident when over 100 pregnant dams sent to another farm in Winona County eventually developed TME, but mink at the recipient ranch did not. This was the first indication that TME was self-limiting and had an incubation period of at least six months. A second TME outbreak occurred simultaneously in several farms in Sheboygan, Calumet and Manitowoc Counties, Wisconsin in 1961.

Fig. 1. A view of typical mink with TME.

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Symptoms were similar to the first outbreak, but the prevalence of TME was limited to 10 to 30% of adult mink among the farms [35]. All of the affected farms were using a ready-mix feed prepared at a common feed plant, demonstrating that TME was likely due to an orally acquired infectious agent. This was followed by another outbreak in 1963 in Sawyer County. A similar pattern emerged with nearly 100% of the 1,100 adult mink affected, but the 4,500 kits did not develop symptoms of TME. There was no evidence of vertical transmission, even to kits that nursed on dams with advanced TME. The term "transmissible mink encephalopathy" was subsequently introduced by Marsh et al. [34] to describe this disease that primarily affects adult mink over one year of age, has an incubation period of at least 6 months, and clinical and neuropathological similarities to scrapie, a fatal neurodegenerative disease in sheep. Fewer than a dozen additional outbreaks of TME have been described in Canada, East Germany, Finland, and the former Soviet Union [1].

The last outbreak of TME was described in 1985 in Stetsonville, Wisconsin, USA [32] and more than 4,300 out of 7,300 adult mink developed TME over a 5-month period, but 600 blue iris kits purchased six months prior to the onset of TME or kits born at this ranch were not affected. This pattern was consistent with previous outbreaks in which exposure to prion/TSE infection took place from 6 to 12 months prior to clinical symptoms, was due to a single exposure of infection, and affected a large number of mink.

Etiology

TME is an orally acquired TSE/prion disease, but the etiology is uncertain. TME was initially thought to be transmitted by consumption of sheep carcasses or by-products due to similarities between the diseases, but only in one case were sheep products (e.g., heads) linked to mink diet. Although it cannot be conclusively demonstrated that sheep products were not the source of TME, the inclusion of sheep products in mink feed was demonstrated only rarely [35]. Experimental inoculation of mink with several isolates of scrapie resulted in a few examples of transmission to mink in which the distribution of spongiform lesions was similar to natural TME [39]. Seven sources of scrapie from the U.K. were intracerebrally inoculated into 24 mink kits, but only one mink developed TME after 22 months. Three U.S. scrapie isolates inoculated into 16 kits resulted in 100% penetrance with incubation periods ranging from 11 to 24 months. While this indicates that mink are susceptible to TME, it also suggests that the isolates tested were not likely to be the origin of TME since incubation periods are typically less than one year in natural TME. This indicated that either a subset of scrapie strains are the causative agent of TME or that there is an additional unrecognized source of TME.

A possible clue was provided during the Stetsonville TME outbreak in which the rancher fed his mink commercial feed (e.g., poultry, fish, cereal) and fresh meat primarily from sick or downer dairy cattle within a 50-mile radius of his ranch [37]. He did not recall including sheep products in his homemade feed ration. Upon reviewing prior TME outbreaks in the U.S. and Canada, in all four cases in which records were available and were not linked to a commercial feed plant, downer cattle were also included in the mink diet. The Stetsonville TME isolate, and subsequently additional TME isolates, were transmitted to cattle by intracerebral inoculation and the Stetsonville TME isolate was the first confirmed case of experimental transmission of a TSE/prion disease to cattle. What was striking was that upon experimental transmission of cattle TME back into mink by the oral and intracerebral routes, the incubation periods were similar to that found for mink passaged TME. Hence, the pathogenicity of the Stetsonville TME agent in mink was not altered upon passage into cattle, suggesting that a previously unrecognized TSE/prion disease in cattle may be the source of TME infection. Additional studies strongly suggest that TME has similarities to L-type BSE in transgenic mice compared to H-type or classical BSE [2]. Since the L-type BSE does not appear to be an infectious form of TSE/prion disease, the proposal by Marsh [35,37] that a rare TSE in cattle may be the source of TME infection seems plausible. This is particularly the case in Wisconsin, which has had the majority of TME in the USA and is a prominent dairy state with aged cattle being a primary source of fresh meat for mink ration. Since mink are a sentinel host it is not surprising that they may have been a key host in amplifying a rare cattle TSE disease. Another possible explanation for the high incidence of TME in Wisconsin is based on the recent identification of a mutation in the prion protein gene in cattle with atypical BSE. There may be cattle breeding stock in Wisconsin that carry a mutation in the prion protein gene that is linked to late onset disease and are also the source of

TSE infection for mink TME outbreaks described in the 1960s and 1985.

To this end, mink were shown to be sensitive to scrapie [23,24]. Of interest, following i.c. inoculation with the UK source of scrapie from a Suffolk sheep only a single animal developed the disease. In contrast, American sources B-834 and B-957 from Suffolk sheep readily transmitted to mink. Also, in another outbreak of TME in Stetsonville, Wisconsin, USA, the affected mink were apparently fed with downer cattle but not scrapie-affected sheep [32], and thus TME may result from BSE transmission from cattle to mink [37]. TME is readily transmitted to cattle [26]. The suggestion that TME may result from transmission from infected cattle but not sheep was supported by recent data on phenotypic similarities of TME in cattle and L-type bovine spongiform encephalopathy (BSE) transmitted to ovine transgenic mice (TgOvPrP4) [2]. To this end, L-type of BSE and TME in TgOvPrP4 presented similar molecular mass of all 3 bands of PrP^d. Unglycosylated PrP^d in L-type BSE, bovine TME and typical BSE has the same molecular mass of approximately 18 kDa in contrast to that of diglycosylated PrP^d species which was lower by 0.5-0.8 kDa in L-type BSE and bovine TME as compared to typical BSE. Furthermore, L-type BSE and bovine TME transmitted to TgOvPrP4 mice presented spongiform change of low intensity but PrP^d was strongly expressed including amyloid plaques. Mink were also susceptible to BSE [44].

Exposure by the oral route was ineffective but Marsh and Hanson [35] cited Gajdusek [20] who, in turn, suggested that not the oral route as such but skin and mucosa abrasion are the real port of entry of the agent. The hypothesis was tested experimentally by subcutaneous inoculation of pastel mink with the *Idaho* source of TME [23]. The infectivity spread from the lymph nodes draining the site of the inoculation (1-4 weeks postinoculation, PI), through the other lymph nodes (98-12 weeks PI, the level $10^{3.0-4.0}$ LD_{50}) to the nervous system stage at 20 week PI ($10^{3.0-4.5}$ LD_{50}) to reach the maximum at approximately 28 weeks PI. The sciatic nerve was first affected. The infectivity was detected in both the blood and the thymus, which suggests blood-borne infection. Analogously to scrapie, when the HY TME agent was inoculated into sciatic nerves, hamsters segregated into 2 groups – with a short and a long incubation time [5]. PrP^d was first detected in the thoracic spinal cord and then spread rostrally toward the cervical cord. In the brain, PrP^d was first detected in the red nucleus, first unilaterally and then bilaterally.

Clinical and neuropathological studies in mink

Clinical description

In the first report by Hartsough and Burger [28], the clinical symptomatology of TME was described in detail. The onset was insidious and animals lost their cleanliness and soiled the boxes with urine and feces. Difficulties in swallowing and eating, excitability and tail arching over the back were noticed. Incoordination followed and typical "*jerky stepping action of the hind legs*" developed along with epileptic seizures and self-mutilation. The clinical course was longer in females (2 to 6 weeks) than males.

Neuropathology and immunohistochemistry

The first description of TME neuropathology was also descried by Hartsough and Burger [28], who noticed neuronal degeneration and spongiform change (Figs. 2-4). Those authors also stressed similarities with scrapie. However, in TME transmitted to aged mink of the Chediak-Higashi genotype, the spongiform change may be minimal or even absent [40].

Eckroade et al. [10] described the topography of lesions in experimental TME and the sequential development of those lesions following intracerebral inoculation with TME. The incubation period was

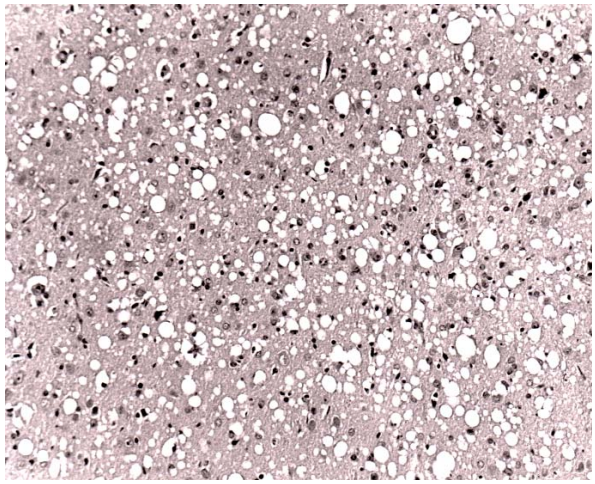


Fig. 2. Spongiform change in natural mink. Courtesy of Dr. William J. Hadlow, USA.

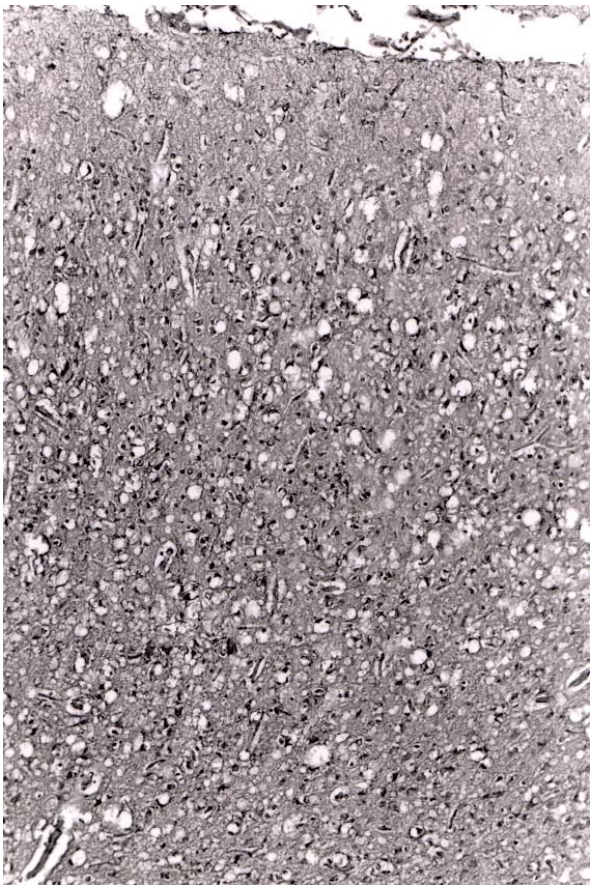


Fig. 3. Spongiform change in natural mink. Courtesy of Dr. William J. Hadlow, USA.

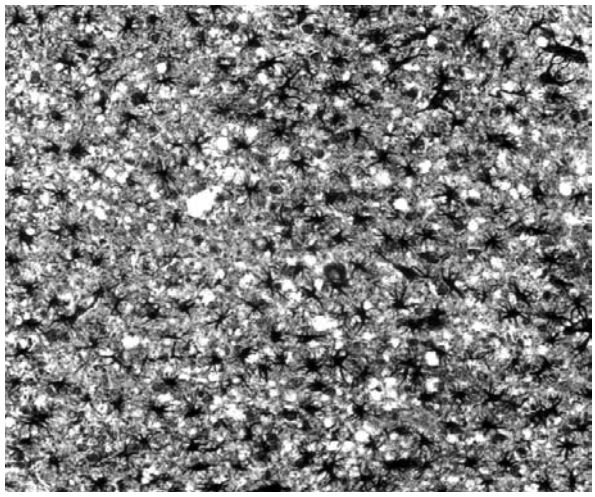


Fig. 4. Astrocytosis in natural mink as visualized by Cajal gold sublimate. Courtesy of Dr. William J. Hadlow, USA.

approximately 31-33 weeks. Spongiform change was severe in the cerebral cortex and an anterior-posterior gradient was observed; the most severe vacuolation was seen in the gyri bordering the cruciate sulcus and within the anterior and the posterior sigmoid gyrus. In the posterior part of the brain, the lesions were minimal. The other parts of the telencephalon were severely affected – caudate nucleus, anterior olfactory tubercle, septal nuclei and putamen – while the globus pallidus was less affected. The diencephalon was affected severely and the hypothalamus was more uniformly vacuolated than the thalamus. The mesencephalon was vacuolated, especially the periaqueductal gray matter. The red nucleus was affected by bizarre intraneuronal vacuoles of great size. The pons, medulla and spinal cord were affected moderately at least.

The earliest spongiform change were observed in the anterior part of the brain 24 weeks postinoculation, sometimes as isolated foci of vacuolation in the cerebral cortex (along the cruciate sulcus) and the thalamus. The caudate nucleus, the periaqueductal gray matter, the central tegmental field of the mesencephalon and the pons were affected minimally at first. Then, spongiform change spread to other neuroanatomical areas; the spreading along the cruciate sulcus was the most rapid.

As already mentioned, from the original Stetsonville TME inoculum, two different strains of TME emerged, i.e. HY and DY, and they differ by topography of lesions [32].The HY strain was characterized by moderate to severe spongiform change in the brain stem, the granule layer of the cerebellum, thalamus, the basal ganglia and the cerebral cortex. In contrast, the DY strain exhibited less severe spongiform change in the brain stem and the cerebellum but more intense vacuolation in the cerebral cortex. The most characteristic lesion of the DY strain in hamsters was focal accumulation of large vacuoles surrounding the pyramidal cell layer of the hippocampus.

The first immunohistochemical studies of TME were published from the laboratory of Gajdusek [2], who used TME-affected mink, TME-infected golden Syrian hamsters and TME-affected squirrel monkeys and ferrets. In sections stained routinely with H & E, typical spongiform change are visible (Fig. 5). Immunohistochemistry for GFAP revealed abundant reactive astrocytic gliosis (Figs. 6-11). Anti-PrP antibodies revealed many different forms of misfolded PrP

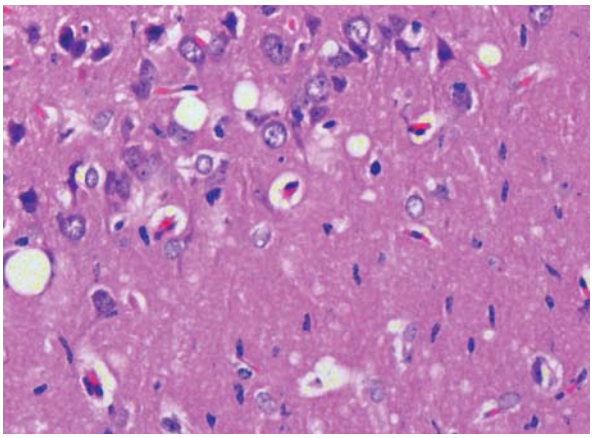


Fig. 5. Typical spongiform change in TME.

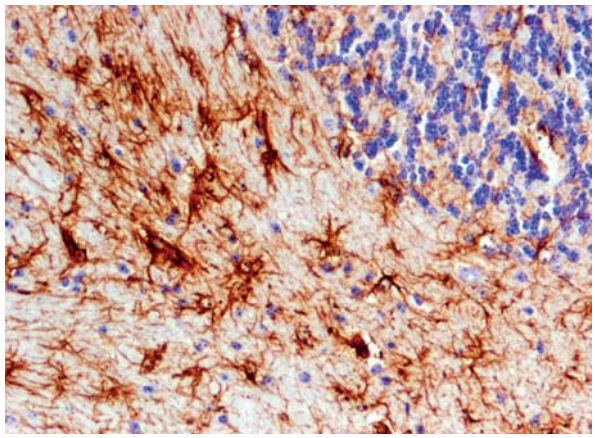


Fig. 6. Abundant reactive astrocytic gliosis as revealed by anti-GFAP staining in the cerebellum.

deposits: plaques (Fig. 9), perineuronal (Fig. 10) and subependymal (Fig. 11) deposits as well as diffuse staining in the cerebellum (Fig. 12) and the hippocampal formation (Fig. 13).

Laser confocal microscopy

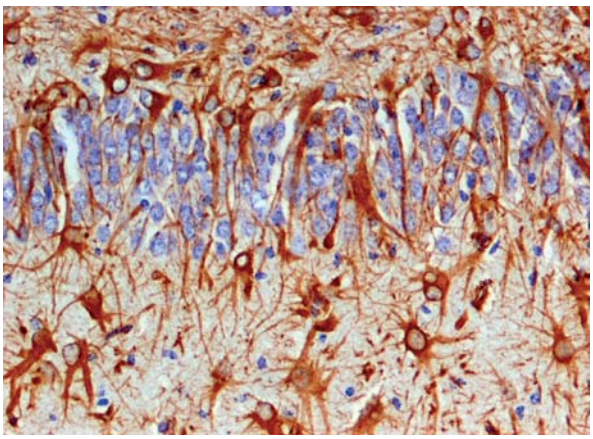
Double labeling for both PrP^{Sc} and GFAP revealed co-localization of both proteins (Figs. 14-15). For immunofluorescent labeling and multichannel confocal microscopy we used mouse anti-PrP monoclonal antibody (clone 3F4, DAKO, Denmark, dilution 1 : 300) and rabbit anti-GFAP polyclonal antibody (DAKO, Denmark, dilution 1:250). The fluorescent-labeled secondary antibody for the anti-PrP was Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, USA, 1 : 200) and for anti-GFAP Alexa Fluor 546 goat anti-rabbit IgG (Molecular Probes, 1 : 200). Immunofluorescence labeling was evaluated using an Olympus FluoView1000 laser scanning confocal microscope.

Transmission to different species

The first transmission of TME from mink to mink using intramuscular inoculation, with the incubation period of 183 to 197 days, was performed by Burger and Hartsough [15,28]. Mink infected orally also developed TME. Neuropathological examination revealed astrocytosis and spongiform change. Of note, using filtration, the size of the infectious agent was estimated to be lower than 500 nm.

TME is transmissible to several mammalian species. TME is transmissible to sheep and goats [23],

A



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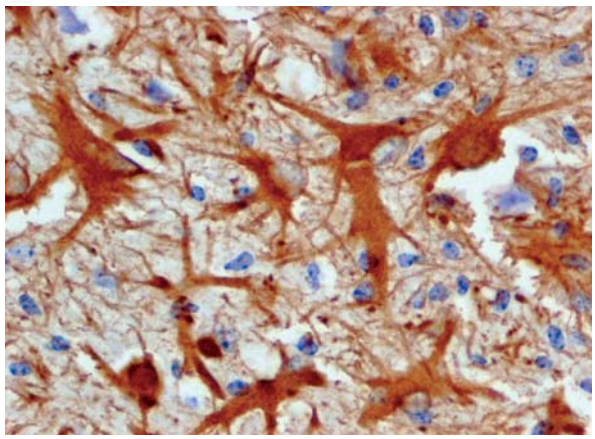

Fig. 7. (A) Low and (B) high magnification of the abundant reactive astrocytic gliosis as revealed by anti-GFAP staining in the hippocampus.

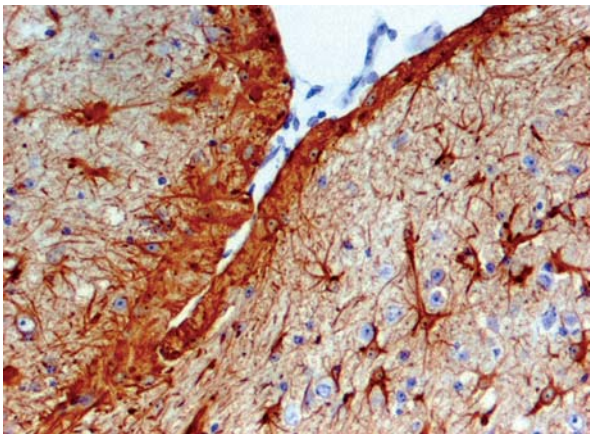

Fig. 8. GFAP-immunopositive astrocytes in the subependymal region.

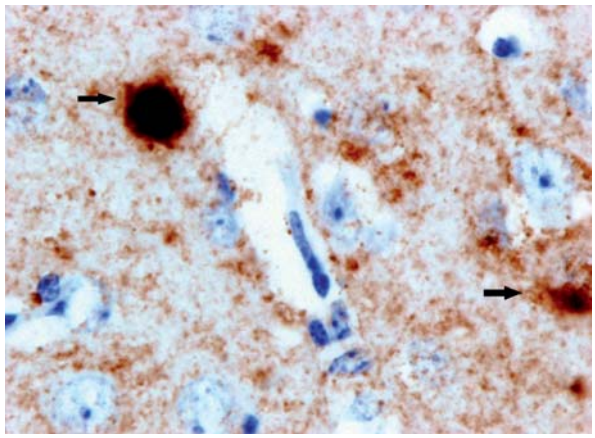

Fig. 9. PrP^{Sc}-immunopositive amyloid plaques (arrows) in the deep cortical regions.

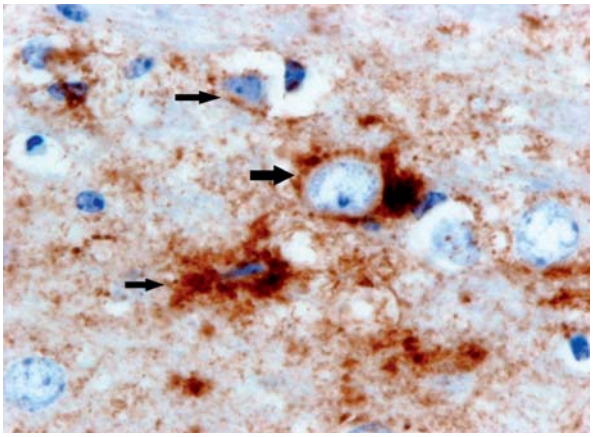

Fig. 10. Perineuronal staining for PrP^{Sc} (arrows) in the deep cortical areas.

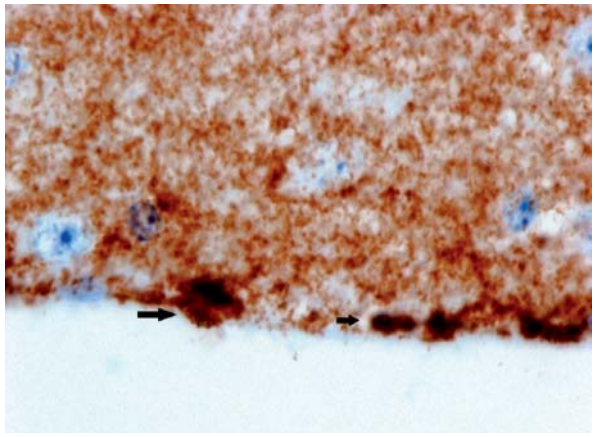

Fig. 11. Subependymal PrP^{Sc}-immunoreactive deposits, some of plaque-like morphology (arrows).

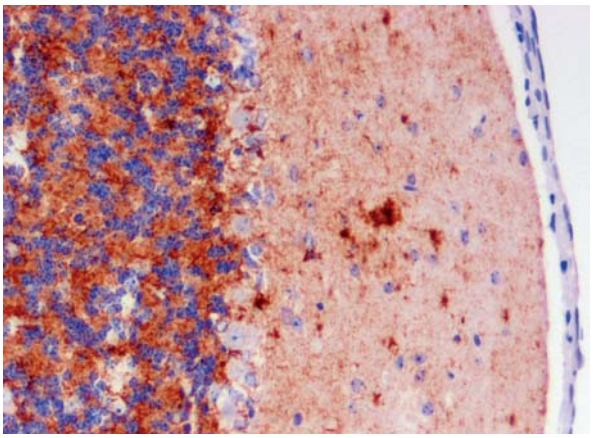

Fig. 12. Diffuse synaptic PrP^{Sc}-immunoreactivity in the cerebellum.

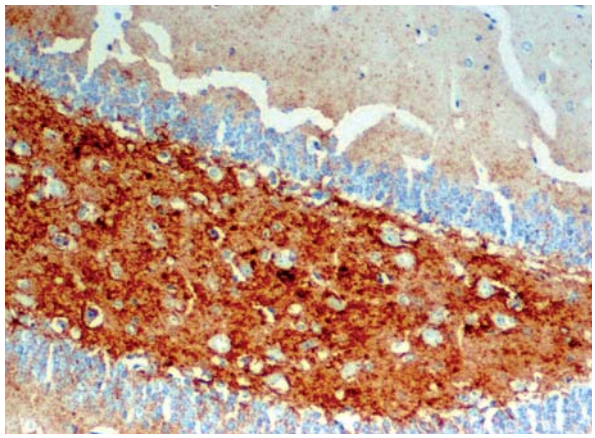

Fig. 13. Diffuse synaptic PrP^{Sc}-immunoreactivity in the hippocampal formation.

hamsters [36,38], skunks, ferrets and raccoons [17,25,27], American sable (pine marten) and beech marten [26], and squirrel monkeys [18]. The transmission of TME from the Stetsonville source to ferrets resulted in a long incubation period of 28 to 38 months on the primary, and 8 to 9 months on the secondary passage [6]. In contrast, TME was never transmitted to mice [10,34,36,45]. However, TME is readily transmitted to transgenic mice with mink *PrP* gene [45]. Kimberlin et al. [29] isolated two strains of TME in Chinese hamsters (333K and 333W) that were readily discriminated by the incubation time (130 and 230 days, respectively).

Cloning of the *PRNP* gene in mink and ferrets

The gene encoding for PrP^{TME} was cloned by Kretzschmar et al. [31]. The open reading frame (ORF) consists of 770 nucleotides (nts) follow by a 3' untranslated sequence of 1650 nt. The deduced mink PrP^{TME} consists of 257 amino acids (aa); the first 24 aa form a signal peptide. There are two Asp glycosylation sites at positions 185 and 201. Of interest, the "anti-PrP" sequence on the anti-sense DNA strand is interrupted by several stop codons, in contrast to the "anti-PrP" sequence of several other species. The closest species of the mustelids belonging to the weasel family are ferrets, whose *PrP* gene is one nt longer than that of the mink gene [6]. There are seven differences between ferret and mink *PrP* gene – 84 (C to A); 231(A to T); 327 (T to C); 354 (T to A), 375 (T to C); 671 (A to G) and 747 (G to A) – but only two differences at the level of aa: 179 (Leu to Phe) and 224 (Glu to Arg).

Molecular biology of TME

The early studies by Marsh et al. [36,41] and others [14] confirmed that the physicochemical properties of the TME agent are similar to those of the scrapie agent. In particular, the TME agent is resistant to formalin: after 4-month exposure the titer dropped from $10^{4.8}$ ip LD_{50}/ml to $10^{6.5}$ ip LD_{50}/ml and to $10^{3.8}$ ip LD_{50}/ml following 20 months.

Molecular basis of TME strain diversity

Experimental transmission of Stetsonville TME into Syrian golden hamsters resulted in the identification of two hamster TME strains upon the third serial passage [33]. One strain had an incubation period of

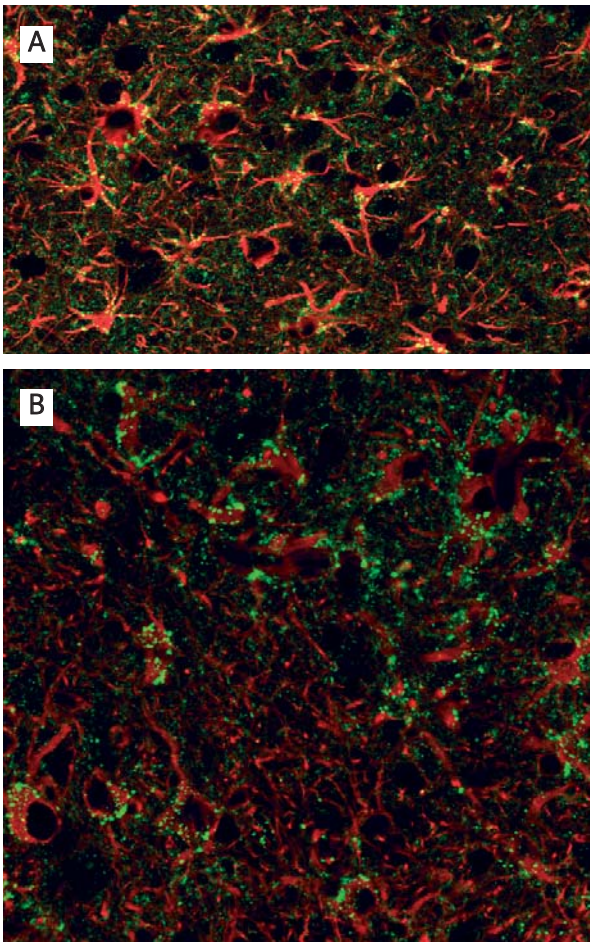

Fig. 14A-B. Laser confocal microscopy double labeling of PrP^{Sc} (green) and GFAP (red) in the hippocampal formation.

65 days and was characterized by hyper-excitability (HY TME strain) and tremor of the head and shoulders, while DY (drowsy) TME strain caused progressive lethargy and drowsiness beginning at 168 days post-inoculation. The HY TME strain replicated to a 100-fold higher titer than the DY TME strain in hamster brain, while following additional serial passage, the DY TME strain remained pathogenic upon passage into mink, while the HY TME strain lost its pathogenicity in mink. These findings were consistent with the isolation of two distinct strains of the TME agent upon interspecies passage in hamsters. Additional studies demonstrated that either both TME strains were present in the original Stetsonville TME isolate, or that the short incubation HY TME strain arose upon passage into hamsters and was preferentially selected for since it can replicate

faster than DY TME. Serial passage of Stetsonville TME into hamsters at a high dilution resulted in isolation of only the DY TME strain, indicating that this was likely the predominant strain isolated from mink.

Although the HY and DY TME strains were consistent with previous studies that have identified two or more TSE strains upon interspecies transmission, the TME strains also provided the first clues as to the molecular basis of TSE strain diversity [8,9,11,12]. The molecular profiles of PrP^{TME} polypeptides revealed a 1-2 kDa shift in molecular weight following limited proteinase K (PK) treatment (21 kDa vs. 19 kDa), which removes the PK-sensitive N-terminal portion. N-terminal sequencing revealed that PK cleaved further into the N-terminus of DY TME compared to HY TME, which suggested that the two TME strains may have distinct conformations. Differences in sedimentation properties and relative susceptibility to degradation with PK were consistent with this hypothesis. Infrared spectra of the TME PrP^{Sc} demonstrated differences in the β-sheet secondary structure content, providing further evidence that these TSE strains had distinct conformations. The ability of these two distinct PrP^{Sc} conformations to self-propagate was also demonstrated in an *in vitro* assay when they were individually incubated with PrP^{C} and the HY TME PrP^{Sc} converted PrP^{C} into a 21 kDa PK-resistant PrP, while DY TME PrP^{Sc} converted PrP^{C} into a 19 kDa PK-resistant PrP [7,30]. In this cell-free PrP conversion assay, the kinetics of PK-resistant PrP formation was also different between the TME strains, which was consistent with a strain-specific pattern of PrP^{TME} formation *in vivo*. Overall, these studies suggested that the molecular basis of TME strain diversity is determined by the strain-specific conformation of PrP^{TME} and that each PrP^{Sc} conformation can convert the same PrP^{C} molecule into a strain-specific subunit of the PrP^{Sc} fibril or aggregate. The formation of each PrP^{TME} conformation may be preferentially favored under specific cellular or subcellular conditions, and the brain distribution of PrP^{TME} may be partially determined by the preferred sites of strain PrP^{TME} formation. Partial evidence for this is provided by the observation that the DY TME strain does not appear to be able to replicate in secondary lymphoid tissue, while the HY TME strain can replicate in lymph nodes and spleen.

Using an *in vitro* PrP^{c} to $PrP^{d\ (TME)}$ conversion reaction [7,10,13,30] it was shown that the conversion is "strain-specific", i.e. HY PrP^{d} only converted PrP^{c} into HY PrP^{d}, and DY PrP^{d} only converted PrP^{c} into DY PrP^{d}. This experiment suggested that certain strain-specificity is encrypted within the conformation of PrP^{d} itself, which, in turn, determines the site of proteinase cleavage and strain-specific size of PrP fragments using Western immunoblot. However, the size of PrP^{d} (either 19 kDa or 21 kDa) is exactly the same as the size of deglycosylated bands purified from human CJD. This may suggest that irrespective of the situation, PrP^{d} may exist only in two major isoforms of 19 kDa and 21 kDa. Whether there are only two strains of every "prion" disease is, in our mind, doubtful.

In a separate study, Mulcahy and Bessen [43] found that conversion of PrP^{c} into PrP^{d} consists of three phases – elongation, depolymerization, and steady-stage phase – and that the elongation phase is that in which strain-specific differences are observed. Those differences between HY and DY strains are the total amount of PrPTME and the time when the reaction peaked. Furthermore, a vast difference in the kinetics of PrPTME accumulation was observed in hamster brains infected with either DY or HY TME strains.

It seems that both DY and HY TME strains are already present as a mixture in the original Stetsonville inoculum and during subsequent passages undergo selection during interspecies transmission [3]. For instance, one of the 4 clones passaged into Syrian golden hamsters bifurcated into a strain characterized by an incubation period ranging from 219 to 522 days and the PrP banding pattern typical of the DY strain of mink, and a second strain with an incubation period of 219 days and PrP pattern that of the DY strain. Upon further passages into hamsters, the incubation periods decreased, the emerging "strain" presented a mixture of HY and DY strains only on subsequent passages, distinct DY strain emerged, and from that, the HY TME strain was selected.

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