Departement of Clinical Research and Veterinary Public Health, Devision of Experimental Clinical Research, Vetsuisse Faculty University of Bern

Director: Prof. Dr. Andreas Zurbriggen

Scientific supervision was provided by

PD Dr. Torsten Seuberlich

Strain-typing of Swiss atypical scrapie isolates in ovine prion protein transgenic mice

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Dorothea Raphaela Götte

Veterinarian

from Wildhaus SG

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PD Dr. Torsten Seuberlich

Bern,

Dean of the Vetsuisse Faculty

University of Bern

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Summary

The pathobiology of atypical scrapie, a prion disease in sheep and goats, is still poorly understood. In a preceding study, we demonstrated that atypical scrapie affected small ruminants differ in the neuroanatomical distribution of the pathological prion protein (PrP^{d}) [1]. To investigate whether these differences depend on host- or rather pathogen- related factors, we transmitted the disease from these animals to ovine prion protein transgenic mice (tg338). The clinical, neuropathological and molecular phenotype in tg338 mice was similar between the Swiss atypical scrapie isolates and a Nor98 control, an atypical scrapie case from Norway. Together with published data, these results suggest that atypical scrapie is caused by a uniform type of prion and that the observed phenotypic differences in small ruminants are likely host-dependant. Strikingly, by using a refined SDS-PAGE technique, we established that the prominent proteinase K resistant prion protein fragment in atypical scrapie consists of two separate unglycosylated peptides with molecular masses of ~5 kDa and ~8 kDa, respectively. These findings show similarities to other prion diseases in animals and humans and pave the avenue towards future comparative directions of research.

Strain-typing of Swiss atypical scrapie isolates in ovine prion protein transgenic mice

Dorothea R. Götte[1], Michaela Gsponer[1], Ingrid Kohler[2], Sylvie L. Benestad[3], Hubert Laude[4], Andreas Zurbriggen[1], Anna Oevermann[1*], Torsten Seuberlich[1*#]

[1]NeuroCentre, National and OIE Reference Laboratories for BSE and Scrapie, Vetsuisse Faculty, University of Berne, Switzerland

[2]Federal Veterinary Office, Liebefeld, Bern

[3]National Veterinary Institute, Department of Pathology, Oslo, Norway

[4]Institut National de la Recherche Agronomique, U892 Virologie Immunologie Moléculaires, Jouy-en-Josas, France

*these authors contributed equally to this study

#corresponding author

DG : dorothea.goette@vetsuisse.unibe.ch

MG : michaela.gsponer@vetsuisse.unibe.ch

IK : ingrid.kohler@bvet.admin.ch

SB : sylvie.benestad@vetinst.no

HL : hubert.laude@jouy.inra.fr

AZ : andreas.zurbriggen@vetsuisse.unibe.ch

AO : anna.oevermann@vetsuisse.unibe.ch

TS : torsten.seuberlich@vetsuisse.unibe.ch

Abstract

The pathobiology of atypical scrapie, a prion disease in sheep and goats, is still poorly understood. In a preceding study, we demonstrated that atypical scrapie affected small ruminants differ in the neuroanatomical distribution of the pathological prion protein (PrP^d) [1]. To investigate whether these differences depend on host- or rather pathogen- related factors, we transmitted the disease from these animals to ovine prion protein transgenic mice (tg338). The clinical, neuropathological and molecular phenotype in tg338 mice was similar between the Swiss atypical scrapie isolates and a Nor98 control, an atypical scrapie case from Norway. Together with published data, these results suggest that atypical scrapie is caused by a uniform type of prion and that the observed phenotypic differences in small ruminants are likely host-dependant. Strikingly, by using a refined SDS-PAGE technique, we established that the prominent proteinase K resistant prion protein fragment in atypical scrapie consists of two separate unglycosylated peptides with molecular masses of ~5 kDa and ~8 kDa, respectively. These findings show similarities to other prion diseases in animals and humans and pave the way towards future comparative directions of research.

Keywords: atypical scrapie, prion, tg338 mice, small ruminants, transmissible spongiform encephalopathy

Introduction

Transmissible spongiform encephalopathies (TSEs), such as scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt-Jakob disease (CJD) in humans, are fatal neurodegenerative diseases which are caused by prions [2]. The neuropathology of TSEs involves spongiform vacuolation, gliosis and the aggregation of a pathological isoform, PrP^{d}, of the host-encoded physiological prion protein, PrP^{c}, in the brain. According to the protein-only hypothesis PrP^{d} is the infectious agent, termed prion [3]. It differs from PrP^{c} by various biochemical properties including partial resistance to proteolytic degradation by proteinase K (PK). PK resistant PrP^{d} fragments (PrP^{res}) can then be demonstrated immunochemically, e.g. by Western immunoblot (WB) [4].

So far, three types of TSE have been found in small ruminants: classical scrapie, BSE and atypical scrapie. Classical scrapie has been known for more than two centuries [5]. It spreads between animals and via contamination of the environment and may cause important losses in affected small ruminant flocks. Experimentally, sheep and goats are susceptible to oral infection with BSE prions [6] and recently two goats have been described that were likely naturally infected in the course of the epidemic in cattle in Europe [7,8]. For this reason small ruminant TSEs have been intensively monitored in European Union member states, but no further small ruminant BSE cases have been identified until today. However, this enhanced surveillance led to the identification of previously unrecognized atypical scrapie cases, first in Norway in 1998 (therefore also termed Nor98) and later in many other countries [9,10]. Such cases revealed discordant phenotypic features, in particular distinct PrP^{res} WB banding patterns and neuroanatomical PrP^{d} distributions as compared to classical scrapie and BSE. Moreover, they were frequently found in

sheep of prion protein genotypes associated with relative resistance to classical scrapie and there was rarely more than one animal per herd affected [11,12]. Upon transmission into laboratory rodent-models and sheep, the distinct phenotype was conserved and it was concluded that atypical scrapie cases are caused by prion types different from those of classical scrapie and BSE [13-17]. Yet, there are still many uncertainties on the pathobiology and the phenotypic diversity of natural atypical scrapie, because in active disease surveillance only limited brain tissues are available.

In a previous study, we had access to whole brains of atypical scrapie affected small ruminants and we found a marked diversity in the neuroanatomical PrP^{d} distribution ([1], table 1). Later, similar findings were reported by other groups in natural cases and after experimental oral transmission of atypical scrapie isolates to sheep [17-20]. Still, whether these differences are attributable to host factors, the involvement of distinct prion types or a combination of both remained obscure.

Herein, we addressed this aspect by transmitting a panel of eight atypical scrapie isolates with different neuroanatomical PrP^{d} distribution patterns in small ruminants into an ovine prion protein transgenic mouse model. This model has been shown to be highly susceptible to classical and atypical scrapie prions [13]. We compared survival times, attack rates, patterns of lesions, PrP^{d} distribution in the brain and biochemical PrP^{res} characteristics between these isolates and with those of classical scrapie, BSE and a Nor98 control. Our results demonstrate that atypical scrapie, despite phenotypic variations in the natural hosts, is caused by a uniform type of prion and also point to a previously unrecognized complexity of the molecular PrP^{res} phenotype.

Materials and Methods

TSE isolates. A total of eight atypical scrapie isolates from Switzerland [1] were included in this study, all of which had been identified by active or passive surveillance for TSEs in small ruminants in the years 2004 and 2005. An atypical scrapie isolate from Norway (Nor98), a classical scrapie isolate from an experimentally infected sheep and a BSE field isolate from a cow (bovine BSE), both of Swiss origin, were included as controls. Further, a medulla oblongata sample of an experimentally BSE infected sheep (ovine BSE) was kindly provided by P. Berthon and F. Lantier from INRA, Tours-Nouzilly, France. Medulla oblongata tissue from a sheep that was tested negative for TSEs by two independent TSE screening tests and by immunohistochemistry (IHC) in different brain structures and lymphatic tissues served as a negative control. Details on all isolates and controls are shown in table 1.

Transgenic mice and bioassay. Tg338 mice express the ovine VRQ PrP on a murine PrP knock-out background at eight- to tenfold levels compared to sheep [13]. Groups of 16 to 20 tg338 mice per isolate were inoculated intracerebrally (ic) at an age of 8 to 10 weeks with 20 µl of 10% [w/v] brain tissue homogenates under isoflurane anesthesia. Mice were monitored for clinical signs at least twice per week and were sacrificed when clinical signs according to end-stage TSE were observed, for animal welfare reasons in case of intercurrent disease or when they reached a minimum survival time of 630 days. At necropsy brains, spleens and hind-limb muscle tissues were collected. The brain of every second animal was fixed in formalin for histopathology and IHC, the others as well as spleen and muscle tissues were snap-frozen in liquid nitrogen and stored at -20°C for Western blot and ELISA analysis. The same set of samples was available from non-inoculated mice of

variable age that served for breeding purposes (n=11). A mouse was considered as TSE positive when PrP^d was detected by Western blot or IHC in brain samples. All animal experiments have been approved by the Committee for Animal Experiments of the Canton of Berne (license #10/08)

Histopathology and lesion profiles. Mouse brains were fixed in 4% formaldehyde, decontaminated for 1 h in formic acid (98%) at room temperature, cut transversely into five pieces as suggested by Fraser and Dickinson [21] and embedded in paraffin. Four-µm thick tissue-sections were stained with haematoxylin and eosin and spongiform lesions were scored in ten defined grey matter areas on a scale from 0 to 5 and in four white matter areas on a scale from 0 to 3 [22].

Immunohistochemistry for PrP^d. The PrP^d IHC was performed on the same mouse brain structures as the lesion profiles at the Institute of Neuropathology, University of Zürich, using a NEXEX robot (Ventana instruments) and monoclonal antibody (mAb) SAF84 (SPI bio, A03208, 1:100) as described elsewhere [23]. The PrP^d deposition pattern was assessed then at the NeuroCentre by microscopic examination of at least six mice per isolate, except of isolate S4/RS for which only one animal was available.

Immunohistochemistry for the astrocyte marker GFAP. After deparaffinization of mouse brain sections the endogenous peroxidase was blocked with 3% [v/v] hydrogen peroxide in methanol and epitopes were demasked by PK (Roche, 5µg/ml) treatment for 15 min at 37°C. Following incubation with 10% normal goat serum for 20 min, GFAP was detected with an anti-GFAP antibody (Dako, Z0334, 1:1000) and stained using the labeled streptavidin-biotin method (LSAB Detection kit, Dako, K5003). The number of astrocytes was counted within one field of view (40x magnification) in the same brain structures as scored for the lesion profiles.

ELISA. Muscle and spleen samples were analyzed in a commercial TSE screening ELISA (Bio-Rad, TeSeE ELISA, 355-1144 and 355-1145). This had previously been evaluated for its capacity to detect PrP^{d} in tg338 brain samples from mice inoculated with atypical scrapie/Nor98, classical scrapie as well as ovine and bovine BSE. Brains of negative control mice scored negative.

Western blot. Whole brains of mice and brain samples of donor sheep and goats were homogenized and purified with a commercial TSE test kit (Bio-Rad, 355-1165) according to the manufacturer's instructions, but with the following modification: the kit-provided PK of unknown concentration was replaced by PK (Roche, 03115828001) at a final concentration of 50 µg/ml and the resulting pellets were resuspended in Lämmli sample buffer (Bio-Rad, 161-0737). PrP^{res} fragments were separated by SDS-PAGE in hand-casted 16.5% tris-glycine gels or in pre-casted 4-20% tris-glycine gels (Invitrogen, EC 6028), immunoblotted onto PVDF membranes (Bio-Rad, 162-0177) and blocked with 1% casein in PBS (Bio-Rad, 161-0783). Three PrP specific mAbs were used: 9A2 ($_{102}$WNK$_{104}$)[24], P4 ($_{93}$WGQGGSH$_{99}$, r-biopharm, R8007), and Sha31 ($_{148}$YEDRYYRE$_{155}$, Bio-Rad TeSeE Western Blot, 355-1169). The binding sites of these mAbs to the prion protein are schematically presented in a previous study [25]. Immunoreactivity was detected using a polyclonal rabbit anti-mouse HRP antibody (Dako, P0260), a chemiluminescent detection system (ECL-plus Amersham Biosciences) and a LAS 3000^{TM} imaging system (Fuji Film, Fuji Inc, Valhalla, NY). Protein bands were quantified and their molecular mass calculated with Quantity One software (Bio-Rad, Version 4.6.2) by comparison with a molecular mass standard (See-Blue, Invitrogen, LC5625).

Deglycosylation. After protein precipitation pellets were resuspended in TD4215 (4% [w/v] SDS, 2% [v/v] β-mercaptoethanol, 192 mM Tris, 5% [w/v] sucrose) and deglycosylated by incubation for 90 minutes at 37°C with PNGase F (New England

Biolabs, P0704L) according to the manufacturer's instructions. Samples were mixed with an equal amount of Lämmli sample buffer and processed in WB as described above.

Statistical analysis. Survival time was defined as the number of days from inoculation to the animal's death. Animals which died within the first days after inoculation were excluded from the study. All statistical analyses were carried out with GraphPad Prism software (Version 5.03). Survival times were statistically compared between groups with the Log-rank Multiple-Comparison Test including Bonferroni-correction ($P< 0.000758$).

Results

Clinical signs of disease. Atypical scrapie/Nor98, classical scrapie and ovine BSE inoculated mice consistently showed characteristic clinical signs of TSE: ataxia and tremor (additional movie). At the terminal stage of the disease the coat got shaggy, animals lost weight and showed kyphosis. There were no obvious differences in the clinical presentation between the isolates. Unexpectedly, most of the negative inoculated control mice (14/18 [n/total]) and some inoculated with bovine BSE (2/15), in which the brain was devoid of disease specific PrP^{d} deposits, showed very similar clinical signs towards the end of their natural lifespan. We therefore defined a mouse as TSE positive, when the disease specific prion protein was detected in the brain by either IHC or WB.

Attack rates and survival times. All eight Swiss atypical scrapie isolates, two derived from goats and six from sheep, and the Nor98 control sample transmitted efficiently to tg338 mice after intracerebral inoculation with attack rates of 100% (table 1). Average survival times in the atypical scrapie groups were very similar (average 188 to 235 days) but significantly ($P<0.0001$) longer for the classical scrapie

control (average 304 days). Mice inoculated with bovine and ovine BSE isolates showed survival times like the negative control group (P>0.5, figure 1). Six mice were killed for other age-related reasons, mostly tumors and in total 4 animals survived without any signs of disease until the end of the experiment (table 1). However, all ovine BSE and 13 out of 15 bovine BSE inoculated mice were PrP^{d} positive in the brain by IHC or WB; two mice were BSE negative, but showed TSE specific clinical signs. Taken together, this suggests that some of the BSE positive mice were not at the terminal TSE stage, but reached the end of their normal lifespan with sub- or preclinical infection. Because of these uncertainties, BSE inoculated groups were excluded from neuropathological comparison.

Histopathological brain lesion profiles. Overall, the intensity and neuroanatomical distribution of vacuolar lesions were very similar in mice infected with sheep (figure 2A) and goat (figure 2B) atypical scrapie isolates and the Nor98 control. However, in the corpus callosum there was a slight difference in the lesion scores between mice infected with the Swiss atypical sheep isolates (figure 3B) and those with the Nor98 control (figure 2A). Also, the goat isolate (G1/RS) resulted in lesion scores similar to the Nor98 control (figure 2B) and the sheep isolate S4/RS in intermediate scores (figure 2A) in this particular structure. For the classical scrapie control, vacuolar lesions were in general milder, with the exception of those in the hypothalamus (figure 2C, figure 3A). In both categories of negative control mice, we observed vacuolar lesions of variable intensity in most investigated structures, notably in the white matter of the cerebellum (figure 2D, figure 3C).

Astrocytic gliosis. An increase in the number of astrocytes indicates a brain injury with loss of neuronal cells, which is characteristic of TSEs. In line with this, atypical and classical scrapie inoculated mice had slightly increased astrocyte counts when compared with those in the negative control mice, independent of the brain structure

and the donor species. By trend, the astrocytic gliosis was correlated to brain structures with higher lesion scores. However, there was no obvious difference between the isolates and the Nor98 and classical scrapie controls (figures 2E-H). Differences between the inoculated and non-inoculated control groups could be related to the intervention and/or to age.

Neuroanatomical PrP^d distribution. We assessed the PrP^d deposition intensity at four brain levels (figure 4A). The PrP^d distribution pattern of all mice inoculated with an atypical scrapie isolate from goats and sheep, including the Nor98 control, where very similar, and strikingly distinct from that in classical scrapie inoculated mice (figure 4B and additional file 2). Another prominent difference between atypical and classical scrapie isolates was the localization and size of plaque-like PrP deposits (figure 5). These plaques were readily observed in HE stained section of classical scrapie but not in atypical scrapie (figure 3A). In mice inoculated with TSE negative tissue we observed a faint intraneuronal staining in the cortex, the septal nuclei and some white matter structures as well as a faint axonal staining in the thalamic nuclei (figure 4B, additional file 2), the latter was also observed in non-inoculated mice (data not shown).

Extraneural PrP^d deposition. Spleen samples were available only of some mice. In classical scrapie (1/1, [positive/tested]), ovine BSE (9/9) and bovine BSE (3/7) affected mice, PrP^d could be detected by ELISA in the spleen. By contrast, those of atypical scrapie/Nor98 (n=4) and negative control mice (n=9) remained negative. We also tested muscle tissue from several mice of all groups, but in none of them the ELISA scored positive.

Biochemical PrP^{res} typing after transmission to tg338 mice. In a first set-up, we analyzed the PrP^{res} WB pattern in our standard mAb P4-based protocol. In order to improve the separation of proteins of molecular masses ~7-10 kDa, as characteristic

for atypical scrapie/Nor98, we used an SDS-PAGE with 16.5% hand-casted polyacrylamid gels. As expected, the PrP^{res} WB patterns in the brains of the inoculated mice were characteristic of classical scrapie, atypical scrapie and BSE and similar to the respective inocula. Interestingly, in some atypical scrapie/Nor98 infected mice we identified an additional PrP^{res} fragment with a molecular mass <6.5 kDa (figure 6A). This prompted us to reanalyze all mouse brains using 4-20% gradient Tris-Glycine gels, which were expected to further enhance the separation of low molecular mass proteins. Indeed, by these means all atypical scrapie as well as Nor98 control inoculated mice showed the same pattern with two distinct bands of lower molecular mass; one migrating at ~5 kDa and a second at ~8 kDa (figure 6B and C).

Representative samples were analysed with mAb Sha31 which binds more C-terminal than P4 and 9A2, however, only the ~8 kDa but not the ~5 kDa PrP^{res} was detected (figure 6D). Enzymatic deglycosylation indicated that fragments migrating at ~5 kDa, ~8 kDa and ~18 kDa correspond to unglycosylated PrP^{res} moieties, whereas those above ~18 kDa are glycosylated. MAb 9A2 resulted in the same pattern as mAb P4, which confirms the bands being PrP^{res} fragments (figure 6E).

Inoculated and non-inoculated negative control mice showed some PK resistant bands but to a different extent. These were in general much less intense and of different molecular mass compared to those in TSE inoculated mice (additional file 3). However, one band was akin to the ~8 kDa band observed in atypical scrapie/Nor98 affected mice in the gradient gels before (figure 6B) and after (figure 6E) deglycosylation.

Biochemical PrP^{res} typing of sheep/goat isolates. We were then interested in whether the dual banding pattern of lower molecular mass was also relevant for atypical scrapie in small ruminants. To this end, we reanalyzed the sheep and goat

tissue homogenates that were used for inoculation with 4-20% gradient tris-glycine gels. Not only did the WB pattern using mAb P4 in atypical sheep and goat scrapie look exactly the same as in atypical scrapie infected tg338 mice, but also no PK resistant peptides were observed in the negative control sheep (figure 7A). Similar to tg338 mice, the ~8kDa peptide but not the ~5 kDa peptide was detected with mAb Sha31 (figure 7B). This clearly suggests that in the natural host, both peptides are disease specific and that these fragments may be C-terminally truncated to a different extent.

Discussion

This study was conducted to dissect host- from pathogen-related factors that impact on the phenotype of atypical scrapie in small ruminants. We show that transmission of a set of defined atypical scrapie isolates of sheep and goats from Switzerland, which displayed distinct neuroanatomical PrP^{d} deposition patterns in the donor animals, resulted in a uniform disease phenotype in tg338 mice with respect to attack rates, survival times, PrP^{d} distribution and the molecular PrP^{res} pattern. In the lesion profiles we observed some subtle differences between the isolates. However, these were in the range of the variability observed between individual mice inoculated with the same isolate and are therefore not considered significant. Thus, the overall phenotype of the Swiss atypical scrapie isolates was indistinguishable from that of Nor98 but clearly different from that of classical scrapie and BSE controls. This corroborates that atypical scrapie involves a prion type distinct from classical scrapie and BSE. Moreover, it clearly indicates that the prion type is identical in the atypical scrapie cases and the Nor98 control under investigation.

The phenotypic traits of the Swiss atypical scrapie isolates in the tg338 model are of striking similarity compared to previously published data on the transmission of

atypical scrapie/Nor98 field isolates from Germany, Norway, France and the UK using the same but also other ovine PrP transgenic mouse lines [13,26-28]. Taken together, these and our findings suggest that there are no major differences between atypical scrapie prions, whether these have been isolated from various geographical origins, from sheep or goats nor from animals of particular PrP genotypes. Our results provide indirect evidence for the observed differences of the neuroanatomical PrP^d distribution in atypical scrapie/Nor98 affected small ruminants being linked to yet unraveled host factors. At this stage further transmission studies in small ruminants will be required to investigate the pathogenesis and host-pathogen interaction in atypical scrapie.

One limitation of the mouse model we used is that mice inoculated with TSE negative brain tissue displayed neurological signs, histopathological lesions (figure 1 and 2) and PrP immunoreactivity at advanced age (figure 3, 4 and additional file 2). This is unlikely the result of a misclassification or cross contamination of the inoculum, because we observed similar lesions and PrP signals in IHC and WB in much younger, non-inoculated mice. In these mice changes were in general milder and neurological signs were not evident. Moreover, the type and distribution of PrP^d deposits in these mice was clearly distinct from that in TSE positive mice. It is well documented for several transgenic mouse lines that overexpression of wild-type and heterologous prion protein may result in neurological disease and CNS vacuolation, especially in elderly mice [29], but this condition was not transmissible. It is therefore conceivable that we observed a similar effect in the tg338 mice. Yet, this situation hampers the interpretation of the disease phenotype after transmission of TSE isolates that reveal long survival times, as is evident for the BSE isolates in the present study. It should especially be taken into account under experimental setups

that aim at confirming presence or absence of prion infectivity by bioassay in prion protein transgenic mice.

The molecular PrP^{res} phenotype in atypical scrapie/Nor98 affected small ruminants and mice has been addressed in detail in a series of studies [14,27,30-33]. Between some of these studies there was a disagreement on the estimated molecular mass of the prominent PrP^{res} band of lower molecular mass (~7-12 kDa), which was likely due to different electrophoresis conditions. However, in all studies this band was considered representing a single PrP^{res} fragment. Using gradient SDS-PAGE and a set of PrP specific monoclonal antibodies, we herein showed that in fact this band involves two distinct PrP^{res} fragments of ~8 kDa and ~5 kDa, respectively.

The ~8 kDa PrP^{res} peptide was detected in atypical scrapie/Nor98 affected tg338 mice, sheep and goats by mAbs P4, 9A2 and Sha31, which indicates that it spans at least from amino-acid positions 93 to 155 of the full-lengths mature prion protein. It was also found in low amounts in negative- and non- inoculated mice and revealed a high resistance to proteolysis, but was absent in TSE negative sheep (additional file 3, figure 7). Therefore, the presence of the ~8 kDa PrP^{res} is not only linked to the pathology observed in atypical scrapie/Nor98, but also to that seen in the negative control tg338 mice. This may be due to the overexpression of the transgene and finally explain the relative high susceptibility of these mice to atypical scrapie compared to other TSEs. By contrast, the ~5 kDa PrP^{res} was found only in atypical scrapie affected tg338 mice and small ruminants and not in the negative controls and healthy small ruminants. Moreover, this peptide did not react with mAb Sha31, and thus it differs from the ~8 kDa PrP^{res} at its C-terminus.

Recently, we reported on a classical scrapie outbreak in a Greek goat flock, in which some of the animals revealed a distinct C- and N-terminally ragged PK resistant PrP^{res} fragment [25]. This peptide showed the same antibody binding properties and

was estimated at a similar molecular mass as observed for the ~5 kDa PrP^{res} in the present study. At that time it remained unclear to which type of prion diseases this peptide was related. The results presented here support that this fragment is rather related to atypical than to classical scrapie, and that both types were present in that flock. A direct comparison of these peptides by gradient SDS-PAGE will be conducted, as soon as material from mouse transmission studies of the Greek isolates is available.

PrP^{res} peptides of low molecular mass have also been described in other types of prion diseases, such as Gerstmann-Sträussler-Scheinker disease [34,35] and Creutzfeldt-Jakob disease [36,37] in humans as well as H-BSE in cattle [38,39]. Future directions of research may therefore focus on a more precise comparative analysis of truncated PrP^{res} peptides and their role in the biology of human and animal prion diseases.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DRG carried out the experiments, collected and analyzed the data and wrote the manuscript. MG performed parts of the Western blot and IHC runs. IK was responsible for the animal experiments, clinical mouse assessment and post mortem sampling. SLB characterized and provided the Nor98 control material and critically revised the manuscript. HL engineered and provided tg338 mice and critically revised the manuscript. AZ participated in the study design and critically revised the experimental setup, data analysis as well as the manuscript. AO provided neuropathological expertise, by assessing the histopathological and IHC data and

critically revised the manuscript. TS conceived and coordinated the study, participated in data analysis and finalized the manuscript.

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Tables

Table 1: Small ruminants isolates, attack rates, clinical signs and survival times in tg338 mice.[#]

Isolates	Species	PrP^d deposition intensity[¶] [Obx/Cbl/Cbr]	PRNP Genotype	Attack rate [N_d/N_i]	Clinical signs [TSE/other/no]	Mean survival time ± SEM [days]
Atypical scrapie						
G1/RS	Goat	1/-/-	AHQ/AHQ	17/17	14/3/0	235 ±6.5
G2/FS	Goat	1/1/3	AHQ/AHQ	14/14	11/3/0	205 ±5.0
S2/RS	Sheep	1/3/-	ARR/ARR	12/12	11/1/0	188 ±4.8
S3/RS	Sheep	1/3/-	ARR/ARR	16/16	15/1/0	227 ±4.2
S4/RS	Sheep	1/-/-	AHQ/AHQ	2/2*	2/0/0	189/256
S5/FS	Sheep	1/1/3	AHQ/AHQ	16/16	15/1/0	216 ±6.0
S6/FS	Sheep	0/3/0	ARR/ARR	14/14	13/1/0	213 ±6.0
S7/CS	Sheep	1/3/3	ARQ/ARQ	14/14	13/1/0	215 ±5.2
Controls						
Classical scrapie	Sheep	3/2/3	VRQ/ARQ	16/16	11/5/0	304 ±17
Nor98	Sheep	1/3/3	AHQ/AHQ	16/16	15/1/0	223 ±3.9
Bovine BSE	Cattle		n.a.	10/15	8/5/2	570 ±13
Ovine BSE	Sheep		ARQ/ARR	17/17	14/1/2	587 ±15
TSE negative	Sheep		n.a.	0/18	14/4/0	567 ±22.4

[#] Abbreviations: Obx, obex; cbl, cerebellum; Cbr, cerebrum; n.a. not available; N_i, number of mice inoculated; N_d, number of mice diseased

[¶] as reported in [1]; -, structure missing; 1, mild; 2, moderate; 3, severe

*for S4/RS fourteen out of sixteen inoculated mice died within two days post inoculation, most probably due to a high bacterial contamination of the sample

Figures

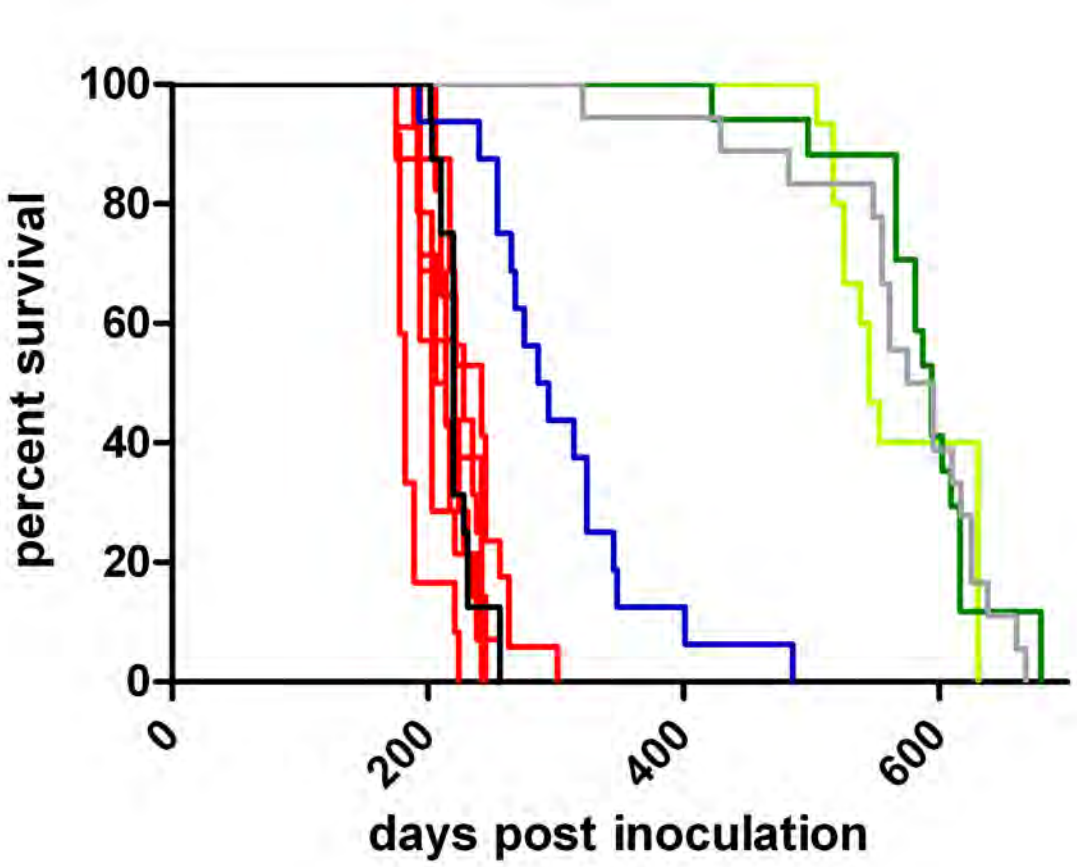


Figure 1. Survival of tg338 mice inoculated with different TSE isolates. Mice infected with Swiss atypical scrapie isolates (red) showed survival times similar to those infected with Nor98 (black), but clearly shorter as compared to the classical scrapie control group (blue). By contrast, ovine BSE (dark green) and bovine BSE (light green) infected mice survived for a time span indistinguishable from the negative control group (grey).

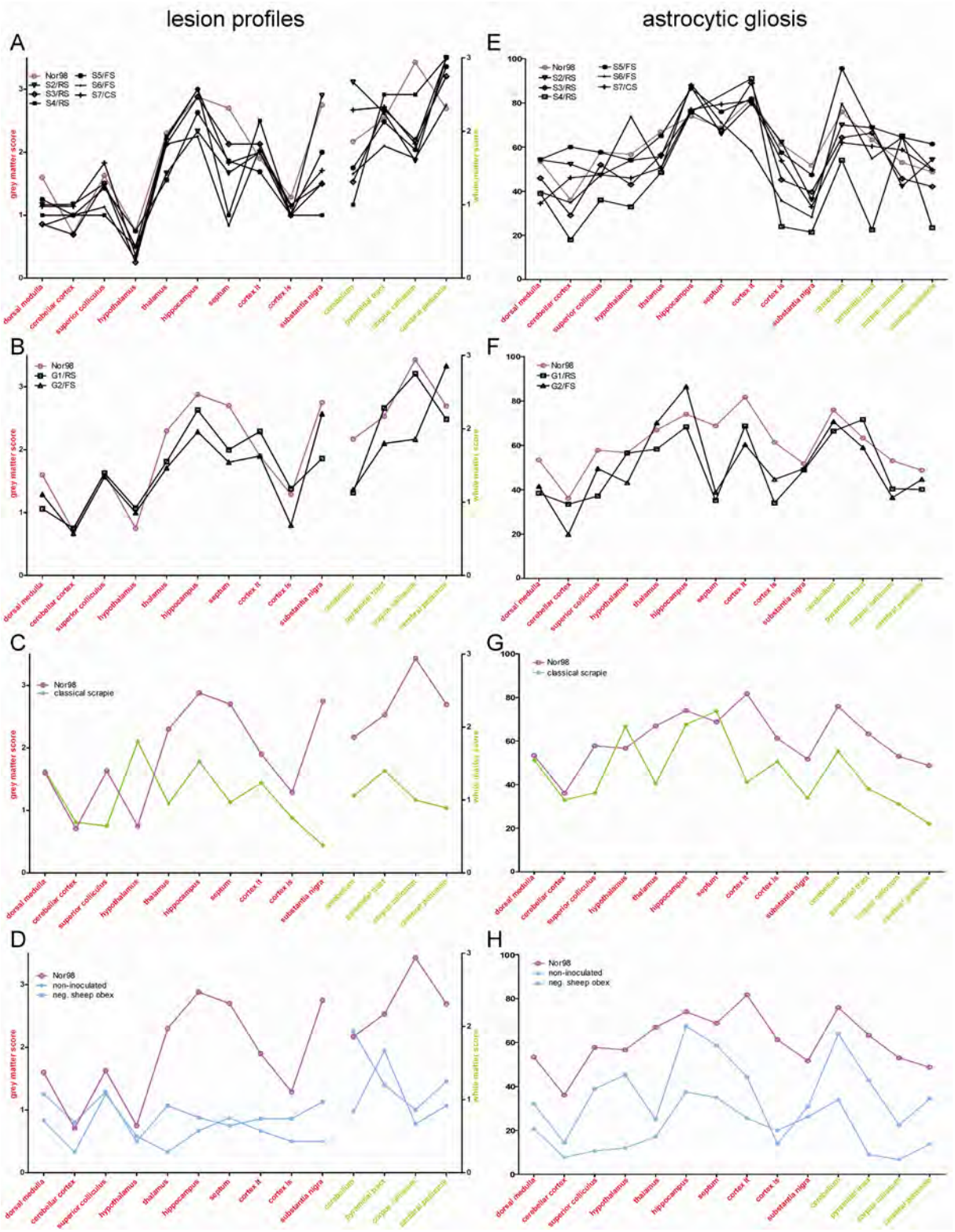


Figure 2. Lesion profiles and astrocytic gliosis scores in scrapie affected tg338 mice. (A-D) Lesions were scored according to the protocol established by Fraser and Dickinson [21] in ten grey matter areas (left y-axis, scale 0 to 5) and in four white matter areas (right y-axis, scale 0 to 3). **(E-H)** Number of astrocytes within one field of

view (40x magnification) in the respective areas. All depicted values represent means of scores in mice inoculated with the same isolate: atypical sheep scrapie **(A, E)**; atypical goat scrapie **(B, F)**, classical scrapie **(C, G)** and negative controls **(D, H)**. To facilitate cross-comparison, Nor98 scores are included in each graph.

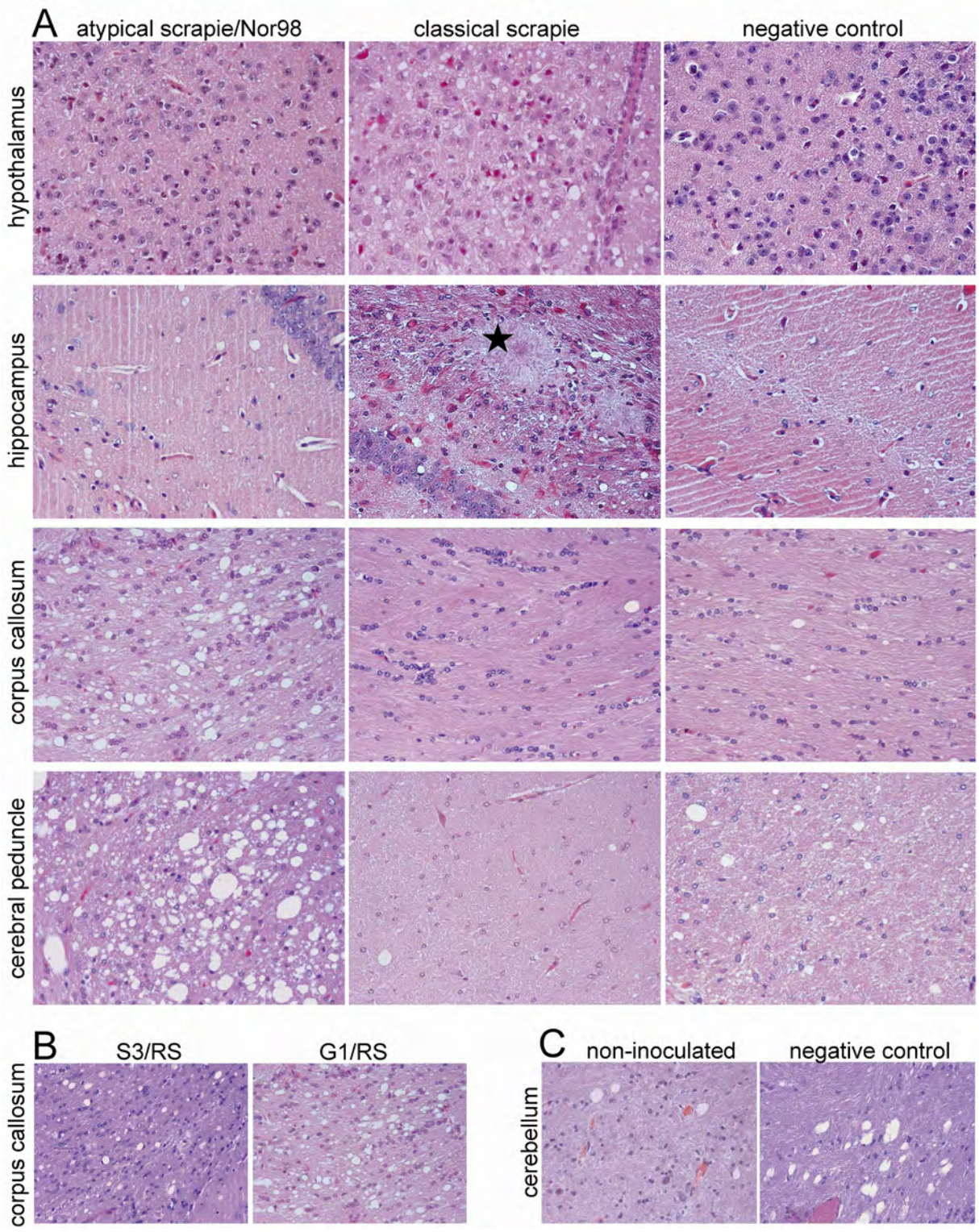


Figure 3. Representative spongiform pathology in tg338 mouse brains. A) Spongiform lesions in the hypothalamus, hippocampus, corpus callosum and cerebral peduncle of TSE inoculated and control mice. Classical scrapie inoculated

mice show amyloid plaques in various brain structures, e.g., in the hippocampus (star). **B)** Variability of spongiform lesions in the corpus callosum between mice inoculated with atypical scrapie/Nor98 isolates. **C)** Cerebellum of two negative control mice showing mild (left) and severe (right) spongiform lesions. All microphotographs are 40x magnifications of HE stained mouse brain sections.

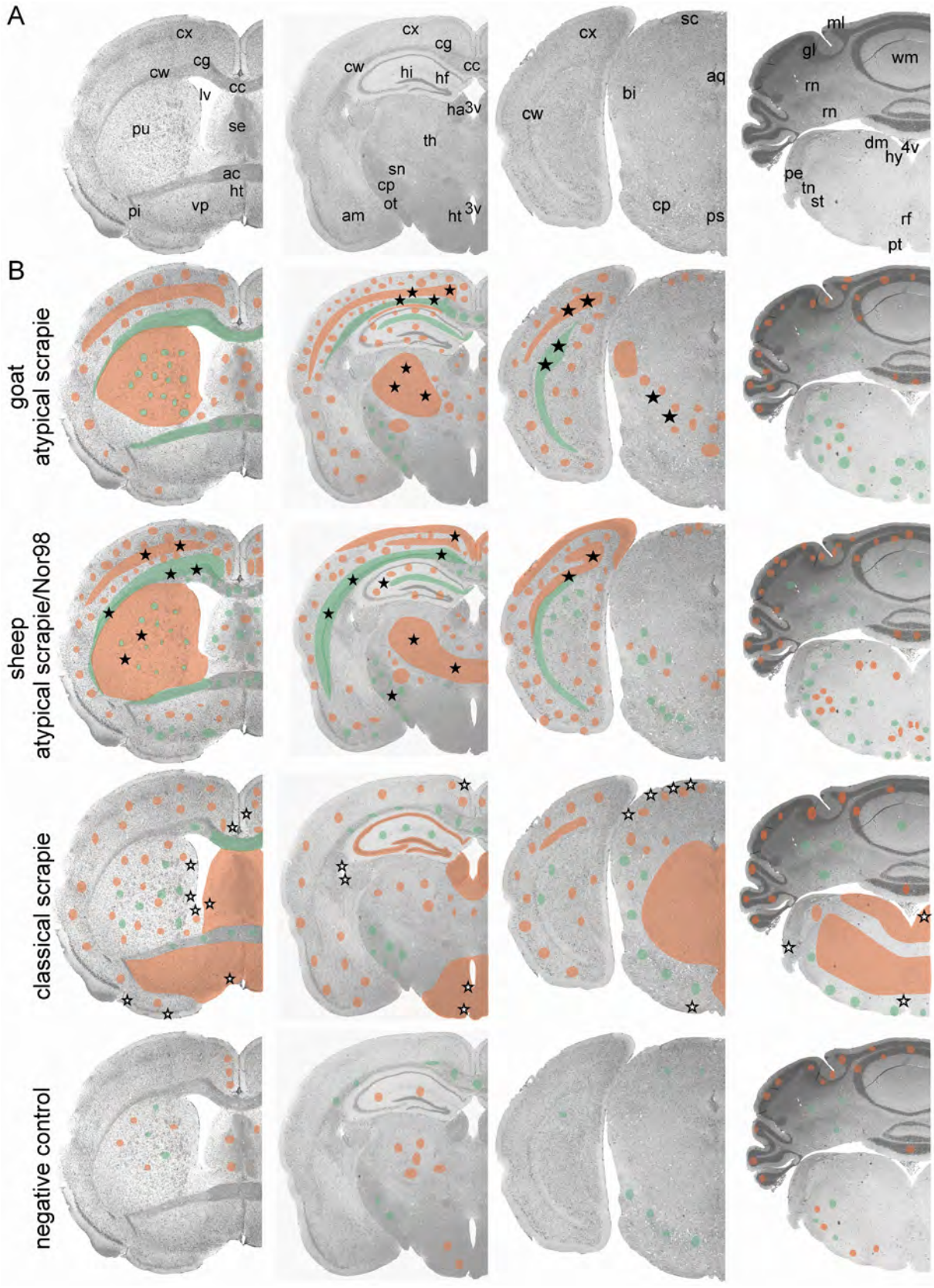


Figure 4. Scheme of the neuroanatomical PrP^{d} distribution in groups of tg338 mice inoculated with different scrapie isolates. **A)** Presentation of the neuroanatomical structures investigated: cortex (cx), cingulum (cg), cerebral white

matter (cw), corpus callosum (cc), caudate putamen (pu), septal nucleii (se), anterior commissure (ac), ventral pallidum (vp), piriform cortex (pi), hypothalamus (ht), lateral ventricle (lv), hippocampus (hi), hippocamal fissure (hf), habenula (ha), 3rd ventricle (3v), thalamus (th), substantia nigra (sn), cerebral peduncle (cp), tractus opticus (ot), amygdala (am), superior colliculus (sc), nucleus brachium inferior colliculus (bi), pons (ps), aqueduct (aq), molecular layer (ml), granule layer (gl), cerebellar roof nuclei (rn), cerebellar white matter (wm), dorsal motor nucleus of the vagus nerve (dm), nucleus hypoglossus (hy), 4th ventricle (4v), cerebellar peduncle (pe), spinal tract nucleus of trigeminal nerve (tn), spinal tract nucleus of trigeminal nerve (st), raphe (rf), pyramidal tract (pt). **B)** PrP^{d} is depicted for mild (dots) and severe (filled areas) deposits in the grey (red) and white matter (green) structures separately. Amyloid PrP^{d} plaques were observed in the neuropil (★) as well as in apparent association to the cerebrospinal fluid space (☆).

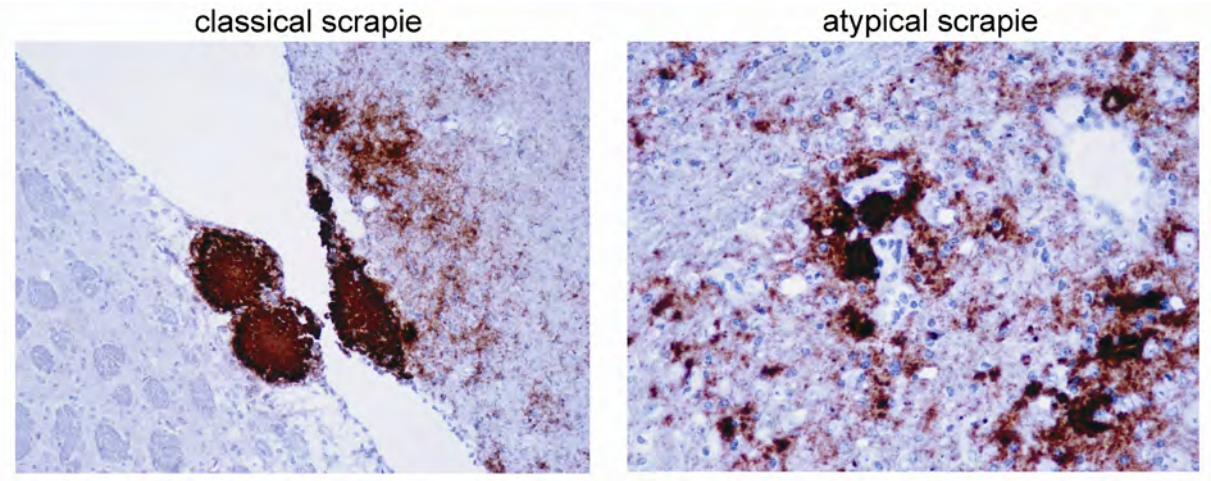


Figure 5. Localization and size of plaque-like PrP deposits in atypical and classical scrapie. In atypical scrapie (40x magnification) relatively small plaques were found in the neuropil, whilst in classical scrapie (20x magnification) plaques were larger, coalescing and always associated to the leptomeninges and the ependyma. PrP^{d} staining was done with mAb SAF84.

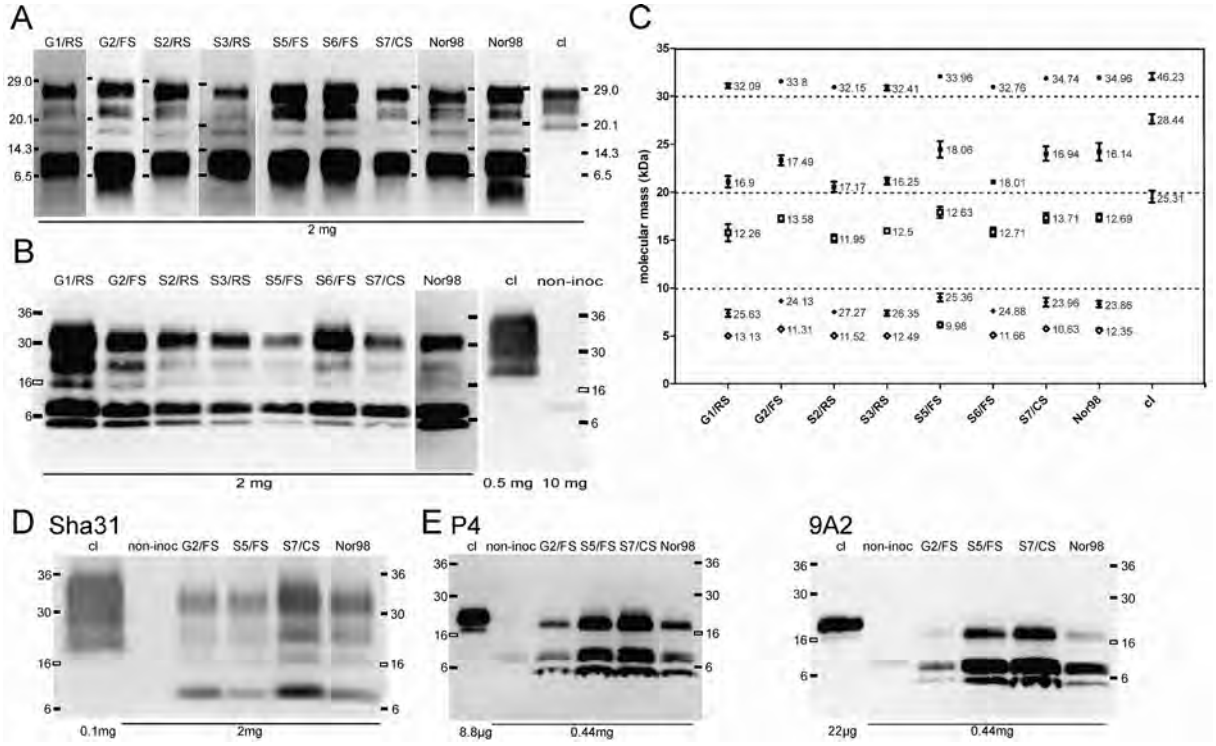


Figure 6. Molecular PrP^{res} typing of atypical scrapie affected tg338 mice.

Western blot for PrP^{res} of representative brain extracts after SDS-PAGE using **(A)** 16.5% tris-glycine gels and **(B)** 4-20% tris-glycine gradient gels (mAb P4).

(C) Molecular masses (Y-axis) and relative quantities of PrP^{res} bands (numbers [%] at the right side of each dot) are depicted as means ±SD of at least five mice per isolate. **(D)** Western blot of PrP^{res} using 4-20% gradient gels and mAb Sha31 **(E)** and after enzymatic deglycosylation with mAbs P4 and 9A2. For the blots, positions of molecular mass standards are indicated at both sides and loaded tissue equivalents at the bottom. Abbreviatons: identification codes for the atypical scrapie isolates are shown on the top; cl, classical scrapie; non-inoc, non-inoculated.

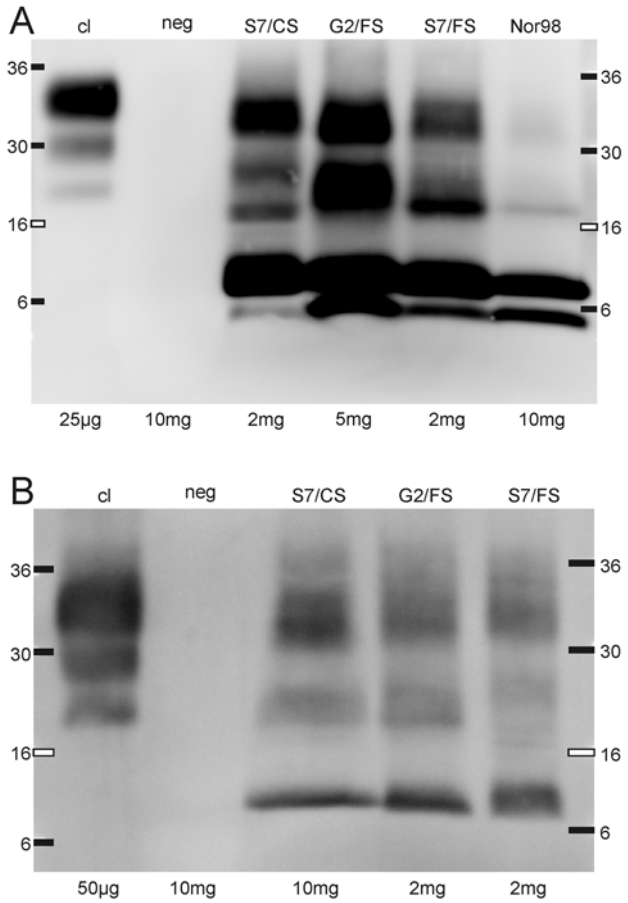
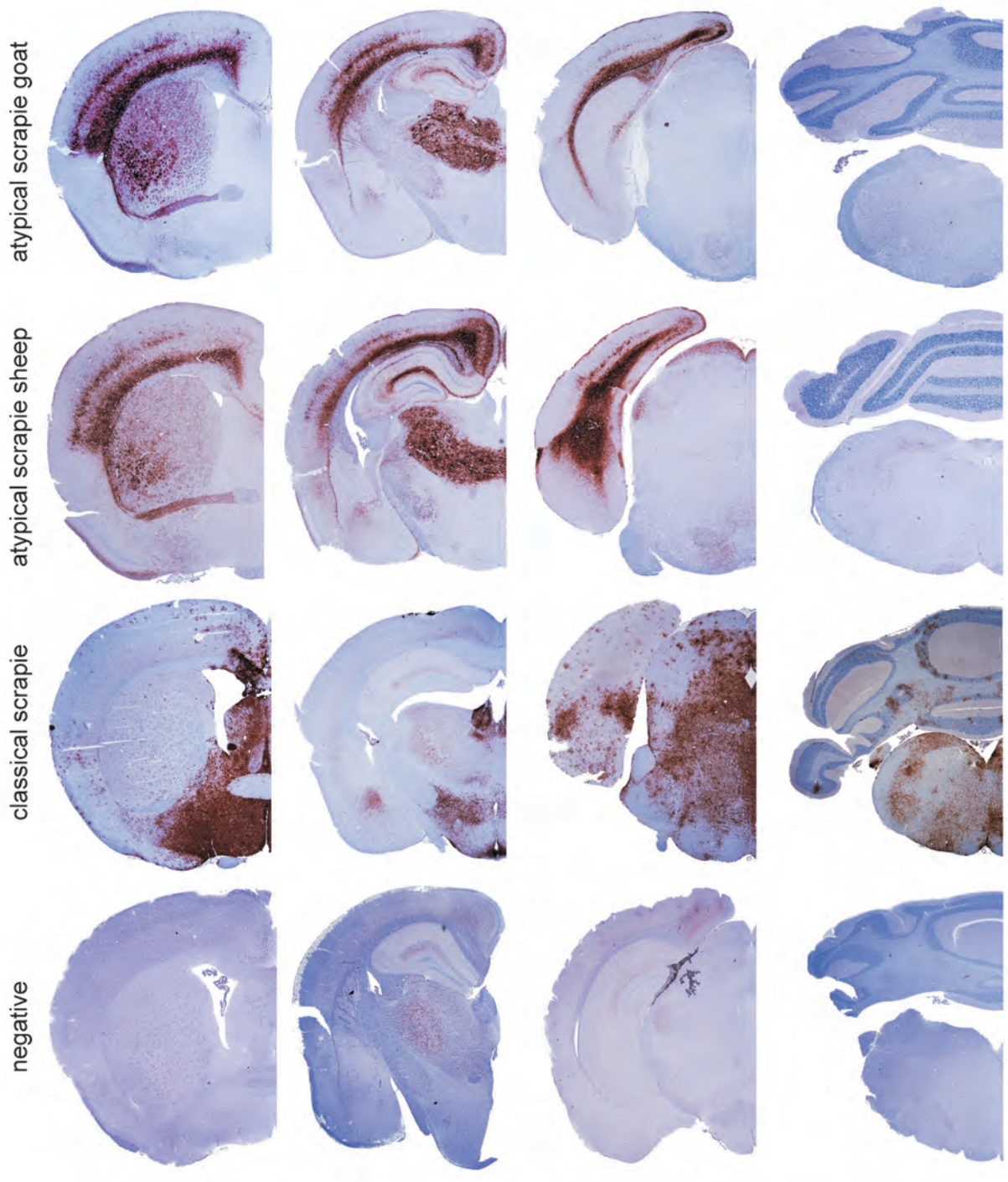


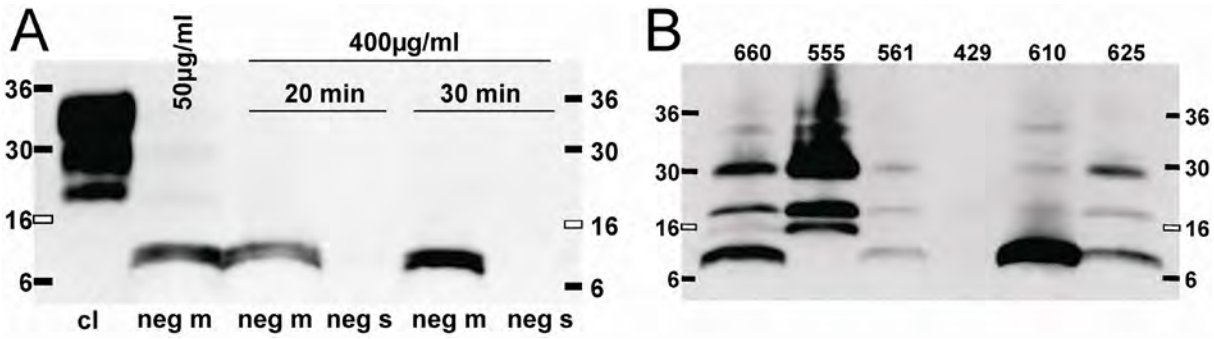
Figure 7. Molecular PrP^{res} typing of atypical scrapie affected sheep and goats. SDS-PAGE was performed in 4-20% gradient gels and the blots were probed with mAb P4 **(A)** and Sha31 **(B)**. Samples of a classical scrapie affected sheep (cl) and a TSE negative sheep (neg) served as controls. Molecular mass standards and tissue equivalents are indicated.

Additional files

Additional movie. file extension: .mpg; **Tg338 mice with TSE specific symptoms**. mice showing gait abnormality and insecurity.



Additional file 2. file extension: .pdf; **Neuroanatomical PrP^d distribution in mice inoculated with different scrapie isolates.** For anatomical structures see figure 4.



Additional file 3. file extension: .pdf; **Western blot banding pattern in brains of TSE negative mice. A)** Non-inoculated mice (neg m) were treated with different concentrations of PK (indicated on top in µg/ml) for different time periods. Upper bands are fully digested when using higher PK concentrations, whereas a ~8kDa band is highly PK resistant. Controls: classical scrapie (cl), negative sheep (neg s). **B)** Tg338 mice inoculated with negative sheep brain show variable banding patterns after treatment with PK (50µg/ml). Survival time in days of individual mice are indicated on the top. Molecular mass standards are indicated at each side. Tissue equivalents loaded per well are 25µg for classical scrapie and 10mg for all other samples.

Curriculum Vitae

Name, Vornamen	Götte, Dorothea Raphaela
Geburtsdatum	25.02.1983
Geburtsort	Arlesheim BL
Nationalität	Schweizerin
Heimatort	Wildhaus SG

08/2002-06/2004	Math.-Nat. Gymnasium Bern-Kirchenfeld, Bern, Schweiz
18.06.2004	Matura; Gymnasium Bern-Kirchenfeld, Bern, Schweiz

09/2004-08/2009	Studium der Veterinärmedizin, Universität Bern, Schweiz
28.8.2009	Abschlussprüfung vet. med. Universität Bern, Schweiz

10/2009-09/2011	Anfertigung der Dissertation unter Leitung von PD Dr. Torsten Seuberlich am Departement für klinische Forschung und VPH der Versuisse-Fakultät Universität Bern Leiter: Prof. Dr. Tosso Leeb

Bern, 27.06.2011

Dorothea Götte

Abstract for Prion 2010

Prion 2010

From Agent to Disease

September 8 - 11, 2010, Salzburg

Molecular Prion Protein Typing in Atypical/Nor98 and Classical Scrapie Affected Transgenic Mice

Dorothea R. Götte[1], Ingrid Kohler[1], Sylvie L. Benestad[2], Hubert Laude[3], Andreas Zurbriggen[1], Torsten Seuberlich[1]

[1]NeuroCentre, National and OIE Reference Laboratories for BSE and Scrapie, Vetsuisse Faculty, University of Berne, Switzerland

[2] National Veterinary Institute, Department of Pathology, Oslo, Norway

[3] Institut National de la Recherche Agronomique, U892 Virologie Immunologie Moléculaires, Jouy-en-Josas, France

A particular feature of atypical/Nor98 scrapie is a Western Blot pattern of the proteinase K (PK) resistant prion protein (PrP^{res}) fragments clearly different from other prion diseases in small ruminants. This pattern consists of a predominant unglycosyaled PrP^{res} peptide that migrates below 12 kDa and multiple bands in the range of 12 -30 kDa, which have been proposed to represent un-, mono-, and diglycosylated moieties of at least one additional fragment. The aim of this study was to investigate by Western blot whether theses fragments are generated by PK cleavage in-vitro or rather by proteolysis in vivo and to identify any qualitative differences in the total of PrP (PrP^{sc+c}) between atypical scrapie, classical scrapie and healthy animals. We analyzed brain tissues of non- inoculated healthy and

terminal stage diseased Tg338 mice that had been inoculated with isolates of atypical/Nor98 (n=4) as well as classical scrapie (n=1). After PK treatment, the inoculated mice revealed the characteristic PrP^{res} pattern of either classical or atypical scrapie. Without PK treatment the total PrP^{sc+c} showed a uniform banding profile in all animals. After deglycosylation we observed N-terminally truncated fragments, which may correspond to previously described PrP-C1 and PrP-C2. However, all these were different from the PrP^{res} fragments in atypical scrapie. Taken together, our results indicate that the PrP^{res} fragments in atypical scrapie are mostly the result of proteinase K cleavage in vitro and not generated in vivo as proposed previously. Still, this situation needs to be confirmed in the natural host.

Poster for Prion 2010

Molecular Prion Protein Typing in Atypical/Nor98 and Classical Scrapie Affected Transgenic Mice

Dorothea R. Götte[1], Ingrid Kohler[1], Sylvie L. Benestad[2], Hubert Laude[3], Andreas Zurbriggen[1], Torsten Seuberlich[1]

[1]NeuroCenter, National and OIE Reference Laboratories for BSE and Scrapie, Vetsuisse Faculty, University of Berne, Switzerland.
[2]National Veterinary Institute, Department of Pathology, Oslo, Norway
3 Institute National de la Recherche Agronomique, UR892 Virologie immunologie Moléculaires, Jouy-en-Josas, France

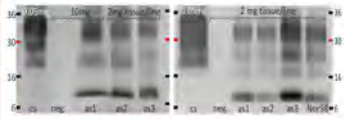
Introduction

A particular feature of atypical/Nor98 scrapie is a Western Blot pattern of the Proteinase K (PK) resistant prion protein (PrP^{res}) fragments clearly different from other prion diseases in small ruminants. This pattern consists of a predominant unglycosyled PrP^{res} peptide that migrates below 12 kDa and multiple bands in the range of 12 -30 kDa, which have been proposed to represent un-, mono-, and diglycosylated moieties of at least one additional fragment. The aim of this study was to investigate by Western blot whether theses fragments are generated by PK cleavage in-vitro or rather by proteolysis in vivo and to identify any qualitative differences in the total of PrP (PrP^{c+sc}) between atypical scrapie, classical scrapie and healthy animals.

Table 1: Small ruminant TSE isolates and bioassay

Isolates	Origin	Species	Genotype	Tg338 mice dead/ inoculated	Incubation period +/- SD [days]
Atypical Scrapie/Nor98					
as1	Switzerland	goat	AHQ/AHQ	16/16	205 +/-20
as2	Switzerland	sheep	AHQ/AHQ	16/16	216 +/-24
as3	Switzerland	sheep	ARQ/ARQ	15/15	214 +/- 19
Nor98	Norway	sheep	AHQ/AHQ	16/16	223 +/- 15
Classical Scrapie					
cs	Switzerland	sheep	VRQ/ARQ	16/19	>304
Negative control					
neg.	Switzerland	sheep	not known	11/19	>525

Fig. 1

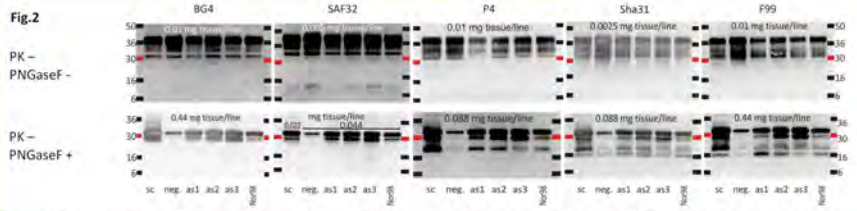


Western blot of small ruminant samples (SR) used for inoculation (A) and samples of tg338 mice inoculated with SR samples (B) both present the characteristic PrP^{res} Western blot pattern for either classical (cs) or atypical Scrapie (as1-3, Nor98) after PK digestion. Loaded tissue equivalents and the molecular mass marker are indicated; antibody Sha31.

Results

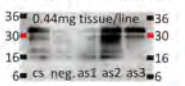
- Three atypical scrapie/Nor98 isolates from Switzerland were efficiently transmitted to transgenic mice that overexpress the ovine VRQ allele (tg338). Incubation periods are similar to a Nor98 control, but clearly distinct from classical scrapie (Table 1).
- The PrP^{res} WB pattern in the brains of inoculated mice were characteristic for either classical (sc) or atypical scrapie (as1-3, Nor98) and had a similar appearance as in the original isolates (Fig. 1). Within the groups all mice showed the same PrP^{res} pattern.
- In non-PK-treated tg338 mouse brain samples we observed a uniform PrP^{c+sc} four-band pattern with bands at ≈ 27kDa, ≈ 31kDa, ≈ 33kDa and ≈ 37kDa (Fig. 2 upper row). There was no difference between inoculated and negative control mice, irrespective of the antibody applied.
- In non-PK-treated and deglycosylated samples from tg338 mice the PrP^{c+sc} WB pattern was similar in atypical/Nor98, classical scrapie and negative controls, but varied depending on the antibody used. The core-binding antibody Sha31 (aa148-155) and the C-terminal binding antibody F99 (aa 220-225) detected four bands of ≈ 16kDa, ≈ 20kDa, ≈ 28kDa and ≈ 31kDa, respectively (Fig. 2 lower row). The ≈16kDa and ≈ 20kDa PrP^{c+sc} fragments did not bind antibodies BG4 (aa54- 57) and SAF32 (aa 62-84). In addition, antibody P4 (aa 93-99) identified the ≈ 20kDa, but not the ≈16kDa fragment. These two fragments are, therefore, likely to be N-terminally truncated but to a different extent. Overall signal intensities in PNGaseF+ samples were slightly lower in the negative controls compared to atypical/Nor98 and classical scrapie affected mice.
- The same four band pattern was also found in the original small ruminant samples when deglycosylated but not PK digested (Fig. 3).

Fig.2



Western blot of non-PK treated mouse samples before (upper row) and after (lower row) deglycosylation with PNGaseF. Banding pattern in atypical (as1-3, Nor98), classical Scrapie (cs) and negative (neg.) inoculated mice are similar. Without deglycosylation a four band pattern is available with all antibodies whereas after deglycosylation the lower fragments are N-terminally truncated. Antibodies used are BG4, SAF32, P4, Sha31, F99

Fig. 3

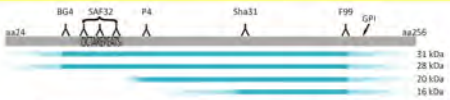


Small ruminant (SR) samples of atypical scrapie (as1-3), classical scrapie (cs) and a TSE negative (neg.) that are deglycosylated but not PK-treated show a uniform four band PrP^{c+sc} pattern; antibody = Sha31.

Discussion

- Mean incubation times, attack rates and the PrP^{res} WB pattern in tg338 mice are similar for the three Swiss atypical/Nor98 scrapie isolates and the Norwegian Nor98 control, but clearly distinct from the classical scrapie control. These findings support the notion that the Swiss and Norwegian isolates involve a similar prion strain.
- The PrP^{c+sc} Western blot pattern in non PK-treated samples before and after deglycosylation shows no qualitative differences between scrapie-affected (cs, as1-3, Nor98) and healthy (neg.) mice. Moreover, the atypical scrapie/Nor98 characteristic band of lower molecular mass (= 7-8 kDa) is not observed in non PK-treated samples. Taken together, this indicates that the PrP^{res} fragment in atypical/Nor98 Scrapie results from PK cleavage *in vitro* rather than from proteolysis *in vivo* as proposed previously.
- Based on our results, we deduce that PrP^{c+sc} in infected as well as PrP^{c} in non-infected mice and sheep is composed of at least three glycosylated fragments (Fig. 4). According to its molecular mass, the 28kDa fragment likely represents the mature full-length PrP^{c}. The 20kDa fragment and the 16kDa fragment are both truncated at their N-termini to a different extend and could correspond to the previously described PrP-C2 and PrP-C1 fragments. Chen S.G. et al. 1995
- While PrP-C2 has been associated to TSE diseased individuals previously, this is not the case in our study. Chen S.G. et al. 1995
- The nature of the 31kDa fragment, however, still remains to be determined.

Fig. 4



Model showing full lengths and proposed N-terminally truncated prion protein fragments in scrapie infected and non-infected tg338 mice and sheep. The grey bar represents the full length PrP^{c}. Monoclonal antibodies BG4, SAF32, P4, Sha31 and F99 are indicated at their approximate binding sites. In green, the four bands found in deglycosylated brain samples of tg338 mice and sheep are indicated together with estimations of their molecular masses.

Materials & Methods

- Tg338 (ovPrP VRQ/VRQ) mice were i.c. inoculated (20µl) with brain tissue homogenates of the following small ruminants TSE isolates: 4 atypical/Nor98 Scrapie (as1-3, Nor98), 1 classical Scrapie (cs) and TSE negative sheep brain; groups of 16 to 20 mice per isolate were inoculated.
- Mice were killed when clinical symptoms of end-stage TSE were observed.
- Whole brain of every second animal was stored in formalin, others were stored frozen and used for histopathological analysis , IHC and biochemical typing respectively.
- Proteinase K (PK) digestion was done according to the TeSeE sheep/goat purification kit but with the use of PK (Roche) in a final concentration of 50µg/ml.
- Undigested brain homogenates are diluted with the same amount of water (v/v) and then they are boiled with the same amount (v/v) of Lämmli SDS PAGE sample buffer.
- For deglycosylation, brain homogenates were purified according to the TeSeE sheep/goat kit but without PK and the pellet then was resolved in TD4215 (4% SDS, 2% β-mercaptoethanol, 192mM Tris, 5% sucrose) and was then incubated for 90 minutes at 37°C with PNGaseF (New England Biolabs) according to the manufacturer's instructions.
- Western blot was done on 4-20% acrylamid gradient-gels with a panel of monoclonal antibodies: BG4, SAF32, P4, Sha31, F99 (binding sites are indicated above)
- Molecular mass marker: See-Blue (Invitrogen)

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