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Short Communication

Characterization of atypical scrapie cases from Great Britain in transgenic ovine PrP mice

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Twenty-four atypical scrapie cases from sheep with different prion protein genotypes from Great Britain were transmitted to transgenic tg338 and/or TgshpXI mice expressing sheep PrP alleles, but failed to transmit to wild-type mice. Mean incubation periods were 200–300 days in tg338 mice and 300–500 days in TgshpXI mice. Survival times in C57BL/6 and VM/Dk mice were >700 days. Western blot analysis of mouse brain samples revealed similar multi-band, protease-resistant prion protein (PrP^{res}) profiles, including an unglycosylated band at ~8–11 kDa, which was shown by antibody mapping to correspond to the ~93–148 aa portion of the PrP molecule. In transgenic mice, the incubation periods, Western blot PrP^{res} profiles, brain lesion profiles and abnormal PrP (PrP^{Sc}) distribution patterns produced by the Great Britain atypical scrapie isolates were similar and compatible with the biological characteristics of other European atypical scrapie or Nor98 cases.

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Scrapie is a transmissible, prion protein (PrP) disorder that causes a progressive degeneration of the central nervous system (CNS) of sheep and goats. Although recognized for centuries, and characterized by the vacuolar pathology the disease produces in the brain, the aetiology of this transmissible spongiform encephalopathy (TSE) as a prion disease only emerged in the 1980s during the biochemical investigation of the nature of the transmissible agent (Prusiner, 1982). Prions are cellular proteins that can transfer metabolic and pathological phenotypes vertically from parent to progeny or horizontally between cells and animals (Wickner, 1996). Scrapie is characterized by the accumulation of the abnormal prion form (PrP^{Sc}) of the normal cellular prion protein (PrP^{C}) in the CNS or peripheral tissues of sheep and goats. Whereas PrP^{C} is completely hydrolysed by proteinase K (PK) under mildly denaturing conditions, a core structure of PrP^{Sc} (i.e. PrP^{res}) resists degradation due to its differing molecular shape and/or degree of aggregation (Hope *et al.*, 1986). Different alleles of the PrP gene are linked to the susceptibility and disease incubation period of an animal naturally or experimentally exposed to prions (Westaway *et al.*, 1987; Goldmann *et al.*, 1990; Hunter *et al.*, 1996) and their relative effects can change depending on the prion type or strain (Goldmann *et al.*, 1994).

Historically, clinical signs, biological transmission properties and brain pathology have been used to classify scrapie isolates, while more recently categorization based on the electrophoretic mobility and immunochemical

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Supplementary figures are available with the online version of this paper.

properties of the prion form of PrP^{C} has developed (Beringue *et al.*, 2008). In 1998, a novel type of prion protein disorder was identified in sheep in Norway and termed Nor98 (Benestad *et al.*, 2003). This atypical form of scrapie is found predominantly in sheep carrying PrP genotypes associated with relative resistance to classical scrapie (Moum *et al.*, 2005; Saunders *et al.*, 2006; Lühken *et al.*, 2007). Atypical scrapie prions exhibit a different protease-resistant (PrP^{res}) core structure compared with the ~27–30, 23–26 and 21 kDa triplet profile of classical scrapie (Hope *et al.*, 1999; Baron *et al.*, 2000; Stack *et al.*, 2002), including identification of a prominent low molecular mass band at ~11 kDa by Western blot analysis (Buschmann *et al.*, 2004; Klingeborn *et al.*, 2006; Gretzschel *et al.*, 2006; Arsac *et al.*, 2007). The disease has also been detected in goats where it was speculated, based on different PrP^{Sc} immunohistochemical (IHC) patterns, that it could be found as several biological types (Seuberlich *et al.*, 2007).

Transmission of atypical scrapie cases from France and Germany, and Nor98 from Norway, showed that the disease could be transferred experimentally to transgenic mice (Le Dur *et al.*, 2005; Arsac *et al.*, 2009; A. B.-B. and M. H. G., personal communication). The purpose of this study was to characterize atypical scrapie cases from sheep identified by active surveillance of British abattoirs during 2002–2003 (Elliott *et al.*, 2005; Everest *et al.*, 2006) and to determine their transmissibility to transgenic and wild-type mice. Tg338 mice [expressing ovine PrP^{VRQ} (Vilotte *et al.*, 2001; Le Dur *et al.*, 2005)], TgshpXI mice (expressing ovine PrP^{ARQ}; Kupfer *et al.*, 2006) and wild-type C57BL/6 ($Prnp^{a}$) and VM/Dk ($Prnp^{b}$) mice (Bruce *et al.*, 1992, 2002) were selected for bioassay to allow comparison of the clinical, biochemical and pathological transmission characteristics of the British cases.

Caudal medulla samples from 24 cases of atypical scrapie from British sheep were prepared as 10 % (w/v) homogenates in saline for inoculation into tg338, TgshpXI, C57BL/6 and VM/Dk mice. Homogenates were treated with ampicillin (1.25 mg ml^{-1}, 24 h, 4 °C), and gentamicin (0.25 mg ml^{-1}) if required, to eliminate bacterial contamination. Heavily contaminated inoculum 9 was heat-inactivated (70 °C, 15 min, twice). The presence of abnormal PrP was confirmed in a sample of each inoculum prior to injection (TeSeE rapid assay; Bio-Rad; Table 1).

Transmission studies were carried out at the Veterinary Laboratories Agency (VLA), the Friedrich-Loeffler-Institut (FLI) and the Institut National de la Recherche Agronomique (INRA) in accordance with national legal, ethical and welfare regulations. Groups of 20 tg338, TgshpXI, C57BL/6 and VM/Dk mice at ~8 weeks old were inoculated with 10 % (w/v) brain homogenates from atypical scrapie and control cases following isoflurane anaesthesia (VLA). Combined intracerebral (i.c.; right parietal lobe; 20 µl) and intraperitoneal (i.p.; 100 µl) inoculations were conducted. Groups of 12 tg338 (INRA) or 15 TgshpXI mice (FLI) were inoculated i.c. only (20 µl). Inoculations of several atypical scrapie isolates, a classical scrapie case, and a sample from a classical scrapie-free flock (Simmons *et al.*, 2009), were duplicated between laboratories for control purposes. Mice showing clinical signs of disease were euthanized and the brains recovered aseptically for confirmatory studies. Brains were hemisected, with one half frozen for Western blot analysis and the other half fixed in formol saline for neuropathology. Incubation period (IP) was defined as the duration in days post-inoculation (p.i.) to termination where transmission was confirmed; survival times were used where no cases of prion disease were confirmed in an inoculated group.

All 24 atypical scrapie isolates derived from sheep of different genotypes transmitted disease to tg338 and/or TgshpXI mice (Table 1). Affected mice developed a progressive ataxia predominantly affecting hind-limb movement. Clinical signs included weight loss, rough coat, pinched face, hunched posture, lethargy and marked affected gait.

In tg338 mice, the mean IP of 11 transmitted atypical scrapie isolates was 222 days p.i. at VLA (mean range 199–233 days) (Table 1). At INRA, the mean IP for a different set of 11 isolates was 245 days p.i. (mean range 220–274 days). Four isolates common to both inoculation sets demonstrated longer mean IPs at INRA by 16–38 days. The most likely explanation is that mice were euthanized at an earlier time point of the clinical phase at VLA.

In TgshpXI mice, the mean IP of eight transmitted atypical cases was 341 days at VLA (mean range 297–390 days) (Table 1). At FLI, the mean IP of 11 transmitted atypical cases was 383 days (mean range 319–482 days). For duplicated isolates, two demonstrated longer IPs at FLI than VLA by 43–75 days, whereas the third produced similar IPs (319 and 329 days, respectively). Although of little bearing on the scientific interpretation of these data, the number of confirmed transmitted cases per inoculation group was lower at FLI than VLA for unknown reasons.

Inter-site variation in IPs and attack rates preclude combining the data from the three laboratories to calculate any significant effects of sheep donor genotype or inoculum PrP^{Sc} content on IPs for these transgenic mouse lines. For the few inocula where a comparison was possible: for the same site, transgenic model and donor genotype, PrP^{Sc} levels in inocula (Table 1, mean ELISA absorbance) were inversely correlated with the IP in transgenic mice. In contrast to the subline data for TgOvPrP4 mice reported by Arsac *et al.* (2009), we did not see a significant effect of sheep donor genotype on IP in tg338 or TgshpXI mice. However, such an effect cannot be ruled out and could be revealed by titration or use of tg338 or TgshpXI sublines with lower expression levels.

At VLA, inoculation of 11 atypical scrapie isolates into C57BL/6 and VM/Dk mice failed to produce a single confirmed case of transmitted disease, with all groups of

Table 1. IP analysis of atypical scrapie cases inoculated into transgenic and wild-type mice

ND, Not done; NA, not applicable. Cases 5, 6, 26, 45, 25 and 24 were inoculated at both VLA and INRA, and cases 7, 27, 45, 25 and 24 were inoculated at both VLA and FLI.

Inoculum	PrP genotype	Mean ELISA OD	tg338 (VLA)		tg338 (INRA)		TgshpXI (VLA)		TgshpXI (FLI)		C57BL/6 (VLA)		VM/Dk (VLA)	
			N/N^{o}*	Mean IP ± SEM†	*N/N*^{o}*	Mean IP ± SEM†	*N/N*^{o}*	Mean IP ± SEM†	*N/N*^{o}*	Mean IP ± SEM†	*N/N*^{o}*	Survival time‡	*N/N*^{o}*	Survival time‡
Atypical scrapie cases														
1	AHQ/AHQ	0.368§	19/20	210 ± 3	–	ND	18/20	327 ± 14	–	ND	0/20	811	0/18	778
2	AHQ/AHQ	0.702§	19/19	210 ± 5	–	ND	14/15	341 ± 14	–	ND	0/18	860	0/20	778
5	AFRQ/AFRQ	0.447	19/20	226 ± 3	12/12	258 ± 8	13/19	390 ± 23	–	ND	0/19	769	0/19	725
6	ARR/AHQ	0.455§	19/20	229 ± 5	10/10	247 ± 12	–	ND	–	ND	0/20	810	0/20	727
7	ARR/AFRQ	0.573	16/16	233 ± 4	–	ND	16/17	316 ± 14	4/15	359 ± 7	0/19	804	0/20	749
8	ARR/ARR	0.761§	19/19	199 ± 2	–	ND	15/15	297 ± 7	–	ND	0/20	852	0/20	742
9	ARR/ARR	0.051	19/19	231 ± 6	–	ND	16/20	346 ± 10	–	ND	0/20	852	0/20	742
12	AHQ/AHQ	0.549§	–	ND	10/10	220 ± 10	–	ND	–	ND	–	ND	–	ND
13	AHQ/ALRQ	0.274§	–	ND	–	ND	–	ND	11/15	438 ± 4	–	ND	–	ND
15	ARR/AHQ	0.132	–	ND	–	ND	–	ND	7/15	349 ± 20	–	ND	–	ND
17	ARR/AHQ	0.128	–	ND	12/12	251 ± 5	–	ND	–	ND	–	ND	–	ND
18	ARR/AFRQ	0.306	–	ND	11/11	223 ± 3	–	ND	–	ND	–	ND	–	ND
26	AHQ/AHQ	0.271	19/20	228 ± 3	12/12	244 ± 6	–	ND	–	ND	0/19	799	0/17	703
27	AHQ/AHQ	0.219	17/20	233 ± 6	–	ND	15/18	329 ± 32	6/13	319 ± 10	0/19	804	0/19	734
34	AHQ/AHQ	1.3	–	ND	–	ND	–	ND	11/15	356 ± 2	–	ND	–	ND
35	AHQ/ALRQ	0.347	–	ND	12/12	239 ± 6	–	ND	–	ND	–	ND	–	ND
36	ARR/AHQ	0.497	–	ND	–	ND	–	ND	5/15	334 ± 28	–	ND	–	ND
37	ARR/AFRQ	0.141	–	ND	12/12	274 ± 9	–	ND	–	ND	–	ND	–	ND
38	ARR/AFRQ	0.895	–	ND	–	ND	–	ND	8/15	341 ± 16	–	ND	–	ND
39	ARR/ARR	1.074	–	ND	11/11	227 ± 9	–	ND	5/14	358 ± 20	–	ND	–	ND
40	ARR/ARR	0.108	–	ND	–	ND	–	ND	12/15	482 ± 33	–	ND	–	ND
45	AHQ/ARQ	0.835	19/19	213 ± 3	11/11	251 ± 7	–	ND	8/14	422 ± 29	0/18	700	0/20	700
46	ARR/ARR	0.465	–	ND	12/12	256 ± 6	–	ND	–	ND	–	ND	–	ND
47	ALRQ/ALRQ	0.505	20/20	229 ± 4	–	ND	19/20	379 ± 16	3/15	454 ± 21	0/20	700	0/20	700
Positive control														
25	ALRQ/VRQ	2.459§	16/16	391 ± 22	9/9	267 ± 3	8/9	519 ± 26	9/15	516 ± 33	4/20‖	622 ± 28	1/19¶	700
Negative control														
24	ARR/ARR	0.020§	3/14	NA	0/12	NA	ND	ND	0/15	675	0/20	891	0/20	797

Table 1. cont.

*N, Number of mice that developed confirmed TSE disease; *N*°, number of mice available for analysis.
†Mean IP (in days); SEM, standard error of the mean.
‡Survival times (in days) of the longest surviving mouse in each inoculated group for atypical scrapie and negative control cases.
§ELISA values for sheep brain homogenate inocula [10 % (w/v) in saline]; values for other sheep brain inocula produced after ultracentrifugation (500 000 ***g***, 4 °C, 30 min) and resuspension at 20 % (w/v) in homogenization buffer (Bio-Rad).
‖Four mice with positive classical scrapie diagnosis.
¶At 700 days p.i., PrP^{res} was detected in one VM/Dk mouse by immunoblotting, in the absence of clinical signs and TSE-associated vacuolation, suggesting preclinical TSE disease.

mice demonstrating survival times of >700 days (Table 1). Furthermore, these wild-type models are also inefficient monitors for natural scrapie and with the classical scrapie-positive control used here exhibited poor attack rates of 4/20 (C57BL/6) and 1/19 (VM/Dk), with a range of IPs of 540–666 and 700 days, respectively (Table 1).

In Western blot analysis detection of atypical scrapie PrP^{res}, frozen brain samples were ribolysed (Omnigene) in homogenization buffer to produce 20 % (w/v) homogenates, which were extracted, treated with PK and analysed according the manufacturer's instructions (TeSeE Western blot; Bio-Rad).

Western blot analysis of brain from tg338 mice clinically affected by Great Britain (GB) atypical scrapie cases showed very similar PrP^{res} profiles, with immunoreactive bands at ~31, 26.6, 24 and 18 kDa as well as a prominent low molecular mass band at ~8–11 (10.6) kDa, as detected by monoclonal antibody Sha31 (Fig. 1, lanes 1–10).

These profiles were similar to or indistinguishable from those of Nor98/atypical scrapie and the French discordant cases transmitted to the same transgenic line and differed considerably from the triple band profile of classical scrapie in sheep (Fig. 1, lane 11) or in TgshpXI or tg338 mice (data not shown). Whereas PrP^{res} was detected by immunoblotting in the spleen of tg338 mice infected with classical scrapie (data not shown), PrP^{res} was not detectable in the spleen of tg338 mice infected with atypical scrapie at terminal disease, which was similar to the findings of Le Dur *et al.* (2005). Western blot PrP^{res} profiles obtained for TgshpXI mouse brain affected by atypical scrapie (data not shown) were essentially similar to tg338 profiles.

To deglycosylate PrP^{res}, PK-treated tg338 brain extracts were treated with methanol (7 vols, −20 °C, 2 h) and centrifuged (13 000 ***g***, 30 min) to precipitate proteins, which were resuspended in deionized water and treated with peptide *N*-glycosidase F (PNGaseF; New England Biolabs), at 37 °C overnight, according to the manufac-

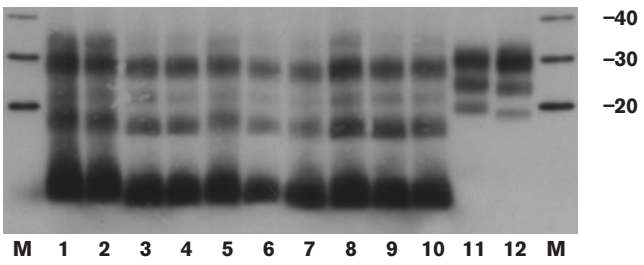

Fig. 1. Western blotting detection of PrP^{res} in brains of tg338 mice inoculated with atypical scrapie from sheep from GB. Lanes 1–10, PK-treated tg338 brains following transmission of atypical scrapie isolates 1, 2, 26, 27, 5, 47, 6, 7, 8 and 9. Lane 11, sheep classical scrapie, +PK. Lane 12, bovine BSE, +PK. M, Molecular mass markers (kDa).

turer's recommendations. Methanol-precipitated pellets of deglycosylated proteins were analysed by Western blotting (as above).

Epitope mapping of atypical scrapie PrP^{res} (before and after removal of *N*-linked glycans by PNGaseF; Supplementary Fig. S1a–i, available in JGV Online) produced by transmission of isolate 6 (ARR/AHQ) to tg338 mice was consistent with the ~8–10 kDa PrP^{res} fragment being a polypeptide of ~60 aa. This fragment is likely to correspond to codons ~93–148 of the ovine PrP gene and agrees with data obtained by others of the fragmentation of atypical scrapie PrP^{Sc} in brains of sheep expressing mainly $AF^{141}RQ$, ARR, AHQ or $AL^{141}RQ$ PrP gene alleles (Klingeborn *et al.*, 2006; Gretzschel *et al.*, 2006; Arsac *et al.*, 2007). As tg338 mice overexpress PrP^{VRQ} and TgshpXI mice overexpress the ALRQ allotype, the similarity of the molecular stability profiles of atypical scrapie PrP^{res} clearly shows independence of the PrP genotype and represents a true strain characteristic distinguishing atypical scrapie from its classical counterpart. PrP^{res} was not detected in C57BL/6 and VM/Dk mice inoculated with atypical scrapie isolates (data not shown), which was consistent with the report of non-transmission of the disease to wild-type mice by Le Dur *et al.* (2005). For comment on the results obtained with negative control inoculum 24 (Table 1) see Supplementary Fig. S2 (available in JGV Online) for details.

Fixed, paraffin-embedded mouse brains were sectioned at the levels of basal ganglia, thalamus, superior colliculus and medulla. Sections were cut and stained with haematoxylin and eosin, according to standard protocols (http://www.defra.gov.uk/vla/science/docs/sci_tse_rl_prp_ihc.pdf) for histopathology and lesion profiling. Vacuolation was assessed semi-quantitatively in nine grey and three white matter brain areas. For each area, the average degree of vacuolar severity from mice inoculated with a specific TSE source was plotted against the corresponding brain area to produce lesion profiles (Fraser & Dickinson, 1968). IHC was performed according to standard protocols (as above) at the same coronal levels as for histopathology, using serial sections, with the detection of abnormal PrP by C-terminal anti-PrP polyclonal antibody Rb486.

Lesion profiles of the atypical scrapie cases derived from >10 clinically affected tg338 mice per inoculation group are presented (Fig. 2a). A greater vacuolation intensity in four grey and white matter areas (G5, G6, G8 and W3) was revealed. These profiles differed from those of classical scrapie in tg338 mice (Thackray *et al.*, 2008) and agreed with published profiles of Nor98 and the French discordant

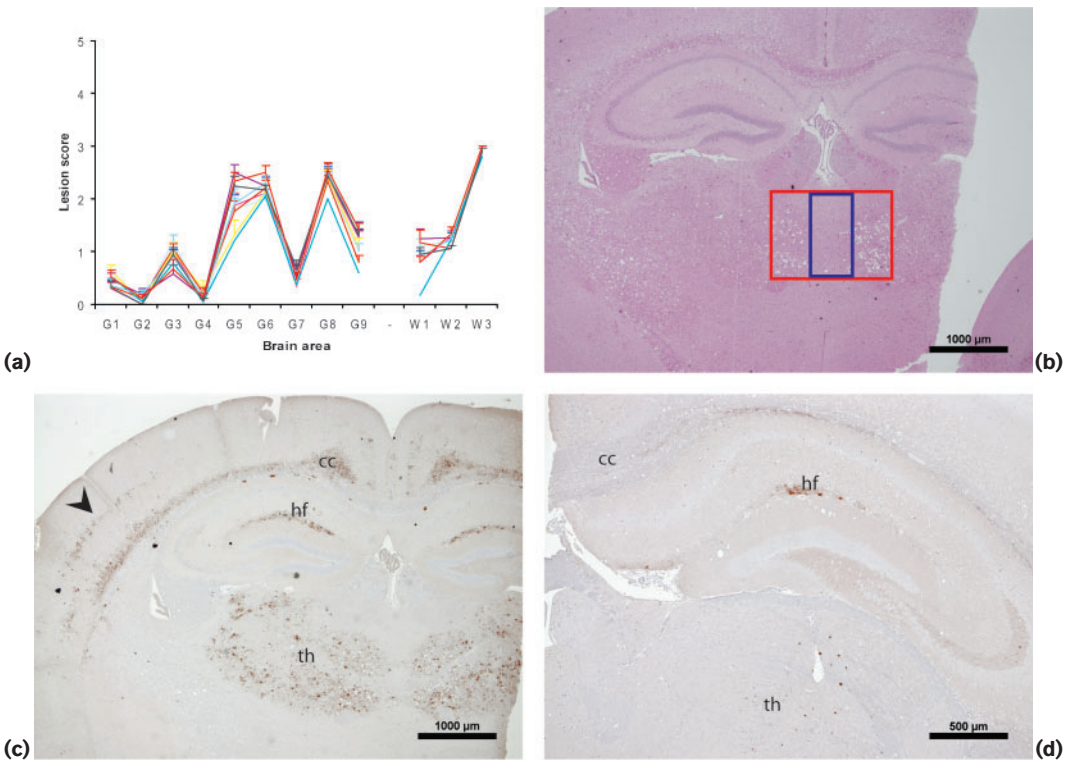

Fig. 2. Lesion profiles and PrP^{Sc} distribution of atypical scrapie cases transmitted to transgenic mice. (a) Lesion profiles of 11 atypical scrapie cases from GB transmitted to tg338. (b) Differences in lesion scoring areas of G5 (thalamus) between VLA (red box, expanded to include medial thalamus) and INRA (blue box; restricted to thalamic midline). (c) PrP^{Sc} distribution in GB atypical scrapie in tg338 and (d) TgshpXI. Arrowhead, cortical layers; cc, corpus callosum; hf, hippocampal fissure; th, thalamus.

cases (Le Dur *et al.* 2005), except for area G5, which was scored significantly higher here. However, it was concluded that the GB atypical cases were histopathologically indistinguishable from French cases and the discrepancy in profiles was attributed to differences in scoring area borders assigned by the two laboratories. As thalamic vacuolation was assessed in a more restricted region at INRA (compared with VLA), differences in area G5 values were the result of methodological differences between laboratories and not of different strains of the agent (Fig. 2b).

In IHC analysis of tg338 mice, comparison of the PrP^{Sc} distribution in coronal brain sections to Nor98 and the French discordant cases revealed that the GB atypical cases (Fig. 2c) were indistinguishable from Nor98 and Nor98-like cases reported from other countries, shown by histoblot analysis (Le Dur *et al.*, 2005). PrP^{Sc} patterns in tg338 mice inoculated at VLA and INRA were also indistinguishable.

In TgshpXI mice, vacuolation, IHC and Western blot analysis were used to diagnose atypical scrapie. IHC analysis revealed that TgshpXI mice inoculated with atypical GB cases had a similar PrP^{Sc} distribution restricted mainly to the hippocampus and cerebellum (Fig. 2d). Compared with tg338 (Fig. 2c), the distribution and intensity of PrP^{Sc} staining in TgshpXI brains were restricted.

In recent years, rapid surveillance testing of healthy slaughter animals in Europe has led to the discovery of apparently novel cattle and sheep prion diseases. During 2002–2003 in GB, biochemical testing of sheep brainstems at slaughter for PrP^{Sc} identified atypical cases of scrapie that had previously evaded detection by the inspection of animals for clinical signs and post-mortem histochemical analysis of the obex. Retrospective analysis of archived tissue samples revealed atypical scrapie cases dating back to the 1980s (Bruce *et al.*, 2007; Webb *et al.*, 2009). However, at the time this study was initiated little was known about the diversity or transmissibility of atypical scrapie, or whether (like variant Creutzfeldt–Jakob disease in humans) cases had emerged as a consequence of cross-species transmission of BSE from cattle to sheep.

IHC mapping of abnormal PrP in Swiss atypical scrapie cases had suggested there was biological variation in the characteristics of this disease in sheep and goats (Seuberlich *et al.*, 2007), and investigations of the biochemical characteristics of PrP^{Sc} from GB cases indicated there could be several molecular types (Everest *et al.*, 2006). Here, we present the transmission of 24 independent isolates to transgenic mice expressing different ovine PrP allotypes, the similarities of these isolates in their biochemical and immunohistological profiles in mouse brain, and their universal failure to transmit to strains of wild-type mice expressing physiological levels of murine PrP. The GB cases present examples of transmissible prion diseases with similar if not identical biology, pathology and biochemistry and our data confirm studies in France (Le Dur *et al.*, 2005; Arsac *et al.*, 2007, 2009) and Germany (Gretzschel *et al.*, 2006) showing that European atypical scrapie cases differ in phenotype from experimental BSE in sheep, behave as if due to a single prion strain and resemble Nor98 (Benestad *et al.*, 2003).

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