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Variant Creutzfeldt-Jakob disease strain is identical in individuals of two *PRNP* codon 129 genotypes

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In 2004, a subclinical case of variant Creutzfeldt-Jakob disease in a *PRNP* 129 methionine/valine heterozygous individual infected via blood transfusion was reported, and we established that the spleen from this individual was infectious. Since host genetics is an important factor in strain modification, the identification of variant Creutzfeldt-Jakob disease infection in a *PRNP* 129 methionine/valine heterozygous individual has raised the possibility that the properties of the variant Creutzfeldt-Jakob disease agent could change after transmission to this different genetic background and concerns that this could lead to a more virulent strain of variant Creutzfeldt-Jakob disease. The variant Creutzfeldt-Jakob disease strain has to date been characterized only in methionine homozygous individuals, therefore to establish whether the strain characteristics of variant Creutzfeldt-Jakob disease had been modified by the host genotype, spleen material with prion protein deposition from a *PRNP* 129 methionine/valine individual was inoculated into a panel of wild-type mice. Three passages in mice were undertaken to allow stabilization of the strain characteristics following its passage into mice. In each passage, a combination of clinical signs, neuropathology (transmissible spongiform encephalopathy vacuolation and prion protein deposition) were analysed and biochemical analysis carried out. While some differences were observed at primary and first subpassage, following the second subpassage, strain characteristics in the methionine/valine individual were totally consistent with those of variant Creutzfeldt-Jakob disease transmitted to 129 methionine/valine individuals thus demonstrated no alteration in strain properties were imposed by passage through the different host genotype. Thus we have demonstrated variant Creutzfeldt-Jakob disease strain properties are not affected by transmission through an individual with the *PRNP* methionine/valine codon 129 genotype and thus no alteration in virulence should be associated with the different host genotype.

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Abbreviations: BSE = bovine spongiform encephalopathy; PrP = prion protein; TSE = transmissible spongiform encephalopathy; vCJD = variant Creutzfeldt-Jakob disease

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Introduction

Variant Creutzfeldt-Jakob disease (vCJD) is an acquired prion disease linked to the consumption of food products contaminated with the bovine spongiform encephalopathy (BSE) agent (Bruce *et al.*, 1997; Hill *et al.*, 1997) and was first reported in the UK in 1996. A peak in deaths was recorded in 2000 and it has since declined with a total of 178 deaths between 1995 and 2018 in the UK (Will *et al.*, 1996; National CJD Research and Surveillance Unit, 2019).

Until 2016, all definite and probable cases of vCJD with genotype data had occurred in the 129 methionine homozygous (MM) genotype suggesting other genotypes may be more resistant to vCJD. In 2016 the first case of clinical vCJD in a 129 methionine/valine heterozygous (MV) individual was reported demonstrating that individuals with other genotypes were susceptible to vCJD and raising the possibility of a second wave of vCJD in individuals of this genotype (Mok *et al.*, 2017). Transmission studies in transgenic mice expressing human prion protein (PrP, encoded by *PRNP*) had predicted that all three genotypes were susceptible to vCJD albeit with differences in susceptibility and incubation period. The 129MV and 129VV genotypes were predicted to have longer incubation periods than in the 129MM individuals and indeed 129MV and 129 valine homozygous (VV) individuals may not develop clinical signs of disease (Bishop *et al.*, 2006). However, since vCJD infection can reside in peripheral tissues there is potential for onward transmission of infection from individuals of all three genotypes (Bruce *et al.*, 2001; Ritchie *et al.*, 2009).

Human to human transmission of vCJD has already been demonstrated and while the majority of vCJD cases are primary cases presumed to be acquired from BSE, three clinical cases of vCJD have been identified in 129MM individuals who had received non-leucoreduced red blood cell concentrates from asymptomatic UK donors (Llewelyn *et al.*, 2004; Hewitt *et al.*, 2006; Wroe *et al.*, 2006). Evidence of misfolded PrP in peripheral tissues has been identified in two other individuals linked to blood and blood products, both of whom were of the 129MV genotype and remained asymptomatic until death from non-vCJD related causes (Peden *et al.*, 2004, 2010). Studies on one of these individuals who had received a red blood cell transfusion from a 129MM donor showed no evidence of abnormal PrP in the brain but deposition in the spleen and a cervical lymph node was observed (Peden *et al.*, 2004) and using protein misfolding cyclic amplification (PMCA), prion seeding potential was demonstrated in a range of tissues (Bougard *et al.*, 2016). Bioassay of the spleen material from this individual also confirmed the presence of vCJD infectivity (Bishop *et al.*, 2013). The other 129MV individual was an adult haemophilic patient who had received factor VIII concentrate prepared from plasma pools known to include a donation from a vCJD infected donor. In this case, spleen material tested positive for the presence of PrP^{res} by western blot analysis (Peden *et al.*, 2010).

Three retrospective studies of anonymized human appendix samples have been carried out in the UK to ascertain the prevalence of vCJD infection (Hilton *et al.*, 2004; Ironside *et al.*, 2006; Gill *et al.*, 2013; Public Health England, 2016). The most recent study identified seven positives out of 15 939 giving a prevalence of ~1:2000 individuals carrying abnormal PrP (Public Health England, 2016). In these three retrospective studies, misfolded PrP has been found in all three codon 129 genotypes.

The single nucleotide polymorphism at codon 129 of the prion protein gene, *PRNP*, encoding either M or V, is recognized as influencing host susceptibility and modifying strain characteristics for human prion diseases such as Creutzfeldt-Jakob disease, Kuru, Gerstmann-Sträussler-Scheinker syndrome and fatal familial insomnia (Lee *et al.*, 2001; Pocchiari *et al.*, 2004; Kobayashi *et al.*, 2015). It was thus recognized that vCJD in different host genotypes may display different strain characteristics.

We have reported previously the infectivity of spleen tissue from an asymptomatic MV blood recipient (MV^R) and the spleen and brain tissue of the MM donor to that individual (MM^D) in RIII and transgenic mice (Bishop *et al.*, 2013). The transmission of MV^R and spleen and brain homogenate of the MM^D into RIII mice also demonstrated differences in incubation times and attack rates between the two inocula suggesting either differences in titre of infectivity or different strain properties between MM^D and MV^R (Bishop *et al.*, 2013). It was therefore important to determine the underlying reason for these differences observed on the initial passage to mice, since strain differences could impact on the transmission potential of the strains.

The MV case demonstrates that there is the risk of transmission between asymptomatic individuals of the MV genotype through blood transfusion or blood products. With the possibility of strain modification and adaptation of the vCJD agent following transmission to a different genotype, it is thus important to assess whether vCJD is modified by the genetic background and whether there are alterations in virulence or pathogenesis. To address this we have carried out an extensive stain typing analysis on the strain of agent found within the spleen of an asymptomatic codon 129MV individual. We have established that the characteristics of this strain are consistent with that of vCJD from 129MM individuals. Thus we have demonstrated that strain properties of vCJD are not altered by the *PRNP* codon 129 genotype of an individual.

Materials and methods

Human tissue selection

Post-mortem tissue (brain and spleen ~0.5–1.5 g) was collected via the MRC Edinburgh Brain Bank, approved by a national

Table 1 Demographic and clinical features of cases included in this study

Characteristics	MV^R blood recipient	MM^D blood donor to MV	MM^{D2008} blood donor to MM	MM^{R2008} blood recipient
MRC reference number	BBN001.34141	BBN_1037	BBN_1028	BBN_1052
Codon 129 genotype	129MV	129MM	129MM	129MM
Patient sex	F	F	M	M
Patient age at illness onset, years	NA	28	23	68
Patient age at death, years	83	28	24	69
Disease duration, months	NA	9	13	13
Case report and further details	Peden *et al.*, 2004; Bishop *et al.*, 2013	Bishop *et al.*, 2013; Urwin *et al.*, 2016	Bishop *et al.*, 2008; Urwin *et al.*, 2016	Bishop *et al.*, 2008; Urwin *et al.*, 2016

F = female; M = male; MV^R = asymptomatic MV blood recipient; MM^D = the MM donor to the MV^R; MM^{D2008} = an MM blood donor; MM^{R2008} = the MM blood recipient to MM^{D2008}.

ethics committee, in line with the Human Tissue (Scotland) Act. Use of human tissue for post-mortem studies has been reviewed and approved by the Sudden Death Brain Bank ethics committee and the Academic and Clinical Central Office for Research and Development (ACCORD) Medical Research Ethics Committee (AMREC). Clinical and demographic details are given in Table 1. Tissue samples were homogenized at 10% (w/v) concentration in sterile physiological saline and stored at −80°C until use.

Mice and experimental design

A panel of three inbred wild-type mouse lines were used for the transmission studies; RIII (also referred to as MR), C57BL6 and VM. RIII and C57BL6 lines are of the *Prnp*^{a} genotype and VM are of the *Prnp*^{b} genotype (Bruce *et al.*, 1991). This combination of mouse lines has been used for the strain characterization of a number of human and animal prion diseases and each line has a characteristic and reproducible incubation period and pathology when inoculated with vCJD.

To characterize the isolates of interest fully, a primary pass (human to mouse) followed by two subpassages (mouse to mouse) were carried out. Subpassage was undertaken from a representative mouse from each mouse line that showed clinical signs and had positive transmissible spongiform encephalopathy (TSE) pathology (TSE vacuolation or PrP deposition) where possible. Brain material from this mouse was then inoculated into the panel of wild-type mice; this process was repeated for the second subpassage.

vCJD infection of mice

All animal studies were carried out in derogated CL3 facilities. The mice were housed in individually ventilated cages under a 12-h light/dark cycle and given food and water *ad libitum*. Cohorts of mice (n = 18–24, 6–8 weeks of age and sex matched) were given prophylactic antibiotics (500 µl streptomycin and 500 IU penicillin) prior to inoculation with human isolates. Mice were anaesthetized with isoflurane and inoculated with 0.02 ml of 10% homogenate via the intracerebral (i.c) route and 0.1 ml via the intraperitoneal route. The MM recipient (MM^{R2008}) CNS was inoculated by the intracerebral route only because of limited tissue availability. For subpassage, inocula were prepared with mouse brain tissue at 1% (w/v) in physiological saline and mice inoculated with 20 µl via the intracerebral route only. Tissue samples were irradiated prior to second subpassage because of an animal house move. Animal studies were approved by The Roslin Institute's Animal Welfare Ethical Review Board and were conducted according to the regulations of the 1986 United Kingdom Home Office Animals (Scientific Procedures) Act.

Scoring of clinical TSE disease and pathology

Mice were scored weekly for clinical signs from 100 (primary pass) or 50 (subpassage) days post inoculation (dpi) by operators blind to isolate/cohort combination according to a previously established TSE clinical scoring system (Dickinson *et al.*, 1968). Mice were scored as unaffected, possibly affected or definitely affected using standard criteria. Any unusual clinical signs were noted and in older animals signs of ageing (loss of body condition, reduced activity) were taken into account. Mice were sacrificed after (i) two consecutive scores of definitely affected; (ii) after receiving scores of definitely affected in 2 of 3 weeks, or (iii) significant deterioration of condition. Mice with no signs of clinical disease were maintained until ~700 dpi or until study termination.

Mice were sacrificed by cervical dislocation, the brains removed and cut sagitally with one half frozen in liquid nitrogen for biochemistry and the remaining half fixed in formal saline for 48 h and decontaminated with formic acid when required, prior to paraffin embedding. Coronal sections were cut (6 µm) and stained with haematoxylin and eosin and TSE-specific vacuolation was quantitatively scored blind to isolate and mouse line by a standard method in nine grey matter and three white matter areas (Fraser and Dickinson, 1968). Mean TSE vacuolation scores were plotted for groups of six mice or more unless otherwise stated.

Prion protein detection by immunohistochemistry

Coronal sections were stained using 6H4 antibody (1:500, Prionics) (Korth *et al.*, 1997) to detect PrP. Antigen retrieval by autoclaving at 121°C in citric acid buffer and a 5-min formic acid (98%) treatment was used. Sections were then blocked with normal rabbit serum prior to incubation with

the primary antibody. Antibody binding was detected with the Vector ABC kit (Vector Laboratories) and visualized with 3,3′ diaminobenzidine chromogen. All sections were counterstained with haematoxylin.

Western blot analysis

Frozen brain samples were homogenized in NP40 buffer [20 mM Tris (pH 7.4), 0.5% v/v NP40 solution, 0.5% w/v sodium deoxycholate] to give a 10% w/v suspension. The suspension was further passaged through a 25 G needle (twice) to break up any remaining aggregates. The homogenate was combined with 3% SDS and treated with 20 µg/ml proteinase K (Novagen) for 1 h at 37°C with shaking.

The prepared sample was suspended in equal volume of Laemmli 2× sample buffer (Sigma-Aldrich) and treated at 100° for 10 min. The products were then loaded onto a 4–12% Bis/Tris gel (Thermo Fisher). After electrophoresis the gel was blotted onto a PVDF membrane. Detection of PrP was with primary antibody 6H4 (Prionics) (Korth *et al.*, 1997) at 1:5000 (for a minimum of 24 h at 4°C with shaking) and an anti-mouse infrared fluorescence, IRDye® secondary antibody (LI-COR) at 1:5000. Images were captured with the LI-COR Odyssey imaging system.

Data and statistical analysis

Animals in which clinical signs were present without pathological (TSE vacuolation and/or PrP deposition) confirmation were removed from the analysis as these signs can also be due to other conditions such as ageing. Animals in which no TSE vacuolation score was available (due to tissue autolysis or technical issues) were also discounted. Early intercurrent deaths (under 200 dpi and 50 dpi for the primary and subpassage, respectively) were excluded from the study. Raw data and samples from previously published studies (Bishop *et al.*, 2008; Bishop *et al.*, 2013) were reanalysed using the criteria above to ensure a valid comparison between all datasets.

Statistical analyses for incubation periods were performed using A Kruskal-Wallis test followed by Dunn's multiple comparisons test using GraphPad Prism 7.0 for windows (GraphPad Software, La Jolla California, USA).

Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Results

In this study, we carried out a characterization study of the MV^R and MM^D (CNS and spleen) at primary passage in our strain typing panel of wild-type mice. This panel consists of RIII and C57BL6 mice, both of the *Prnp*^{a} genotype and VM mice, *Prnp*^{b} genotype. This combination of mouse lines gives highly reproducible and characteristic incubation periods (time between inoculation and death with clinical signs), incubation period rankings (order in which mouse lines succumb to disease) and neuropathology (TSE vacuolation and/or PrP deposition) when inoculated with the BSE or vCJD prion strain. These results were compared to a previously published transmission of brain homogenate from a MM blood donor (MM^{D2008}) and the associated MM blood recipient (MM^{R2008}) (Bishop *et al.*, 2008). We then carried out a second passage of MV^R, MM^D (CNS and spleen), MM^{D2008} and MM^{R2008} and finally a third passage of the MV^R, MM^D (CNS) and MM^{D2008} to allow stabilization and full characterization of each strain.

Primary passage identified differences between isolates

The inoculation of MV^R (spleen) and MM^D (spleen and CNS) to the C57BL6 and VM mice resulted in positive transmission with both clinical disease and/or TSE vacuolation present in all isolate/mouse line combinations (Table 2). This data were then analysed and compared with the previously reported MV^R and MM^D RIII transmission and the MM^{D2008} and MM^{R2008} data set.

On primary passage there were differences in attack rates for clinical signs and TSE vacuolation between the isolates with lower rates observed in both the MV^R and MM^D (spleen) across all mouse lines when compared to the MM^D (CNS). In particular, there was only one VM mouse scoring as clinically positive with no TSE vacuolation (PrP deposition was present) from the MV^R, compared to 47% with TSE vacuolation from the MM^D (spleen) and 54% with TSE vacuolation in previously-reported spleen transmissions from MM individuals (Ritchie *et al.*, 2009).

Incubation periods were calculated for each line and isolate combination for mice exhibiting both clinical signs and TSE vacuolation and the incubation period ranking compared between isolates and those of previous transmissions (Fig. 1A). Incubation periods were increased for both MV^R and MM^D (spleen) in the RIII, C57BL6 and VM mice when compared to those of the MM^D (CNS). The incubation period ranking for the MM^D (CNS) was of the order RIII < C57BL6 < VM mice, similar to vCJD transmissions reported previously. A full incubation period ranking was unavailable for the MV^R due to the absence of TSE vacuolation in the VM mice, this was also true of the MM^D (spleen) where no clinical signs were apparent in the C57BL6 mice.

TSE vacuolation profiles for each line of mice were generated where mouse numbers were sufficient and then compared. C57BL6 mice inoculated with MM^D (CNS) demonstrated similar targeting to that of a typical 129MM CNS transmission (Fig. 1B) with vacuolation targeted to the medulla (G1) and hypothalamus (G4) regions. MM^D (CNS and spleen) showed similar targeting in VM mice to typical 129MM transmissions with peaks in vacuolation intensity at the medulla (G1), thalamus (G5) and septum (G7) regions although an additional peak was observed in the MM^D (CNS) transmission in the superior colliculus (G3), hypothalamus (G4) and thalamus (G5)

Table 2 Incidence of clinical disease and TSE vacuolation wild-type mice challenged with vCJD cases

Isolate	Mouse strain					
	RIII		C57BL6		VM	
	Clinical signs	TSE vacuolation	Clinical signs	TSE vacuolation	Clinical signs	TSE vacuolation
MV^R (spleen)	11/20 (55%)	11/20 (55%)	2/22 (9%)	5/22 (23%)	1/20 (5%)	0/20 (0%)
MM^D (CNS)	18/28 (64%)	21/28 (75%)	11/21 (52%)	18/21 (86%)	13/21 (62%)	18/21 (86%)
MM^D (spleen)	5/20 (25%)	3/20 (15%)	0/21 (0%)	1/21 (5%)	4/19 (21%)	9/19 (47%)
MM^{D2008} (CNS)	21/23 (91%)	21/23 (91%)	14/23 (61%)	21/23 (91%)	20/24 (83%)	21/24 (88%)
MM^{R2008} (CNS)	18/22 (82%)	18/22 (82%)	0/14 (0%)	2/14 (14%)	15/22 (68%)	18/22 (82%)

MV^R = asymptomatic MV blood recipient; MM^D = the MM donor to the MV^R; MM^{D2008} = an MM blood donor; MM^{R2008} = the MM blood recipient to MM^{D2008}.

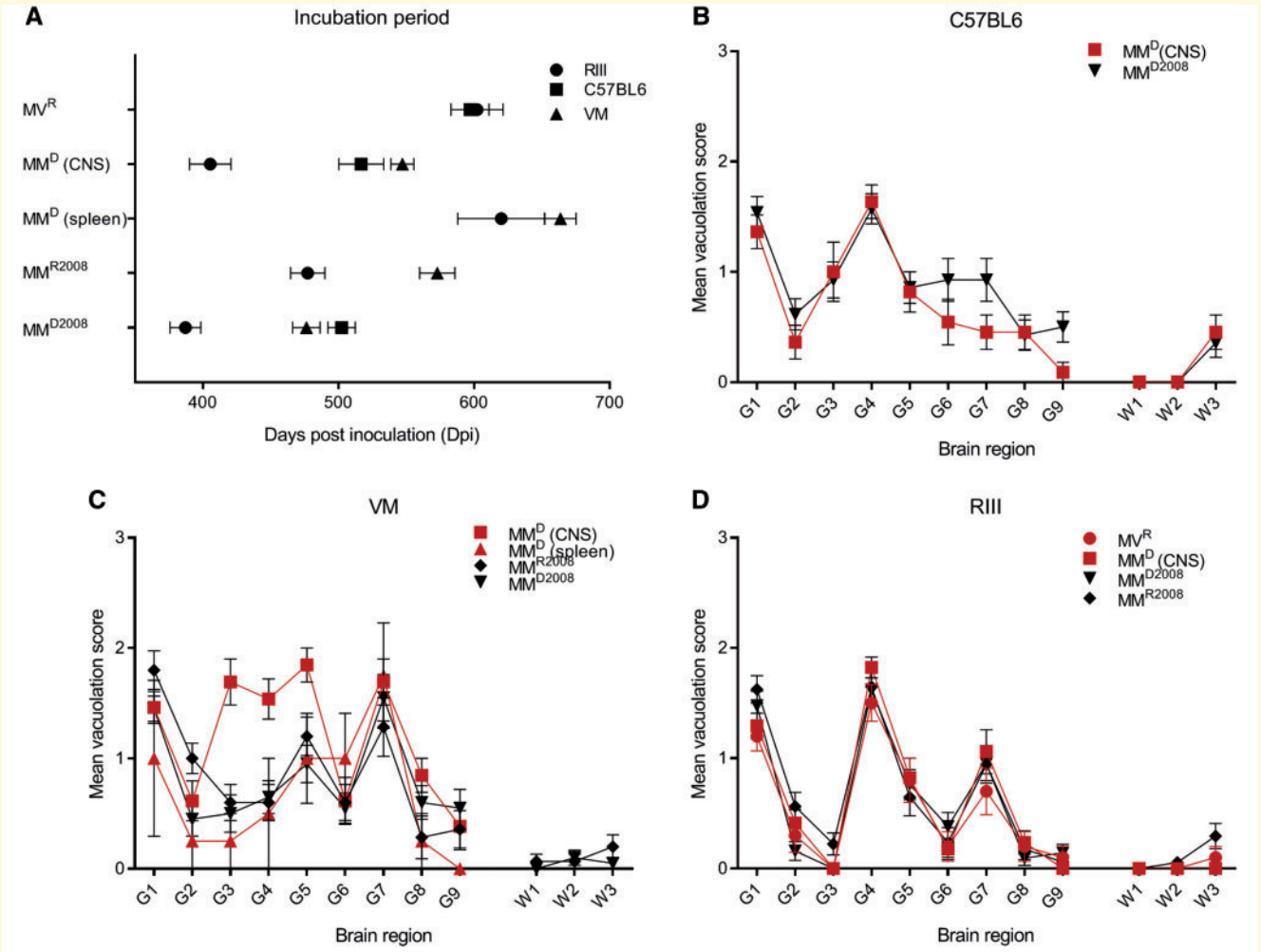

Figure 1 Comparison of incubation periods and TSE vacuolation profiles in wild-type mice challenged with brain and spleen homogenates from four cases of vCJD. The four cases used to challenge wild-type mice were: an asymptomatic MV blood recipient (MV^R), the MM donor to that individual (MM^D), an MM blood donor (MM^{D2008}) and the associated MM blood recipient (MM^{R2008}). (**A**) Incubation periods in RIII, VM and C57BL6 mice. Incubation periods were calculated in mice showing clinical and pathological signs of TSE disease. (**B**) TSE vacuolation profiles in C57BL6 mice ($n \geqslant 6$). (**C**) TSE vacuolation profiles in VM mice [$n \geqslant 6$ in MM^D (CNS), MM^{R2008} and MM^{D2008}, $n = 4$ in MM^D (spleen)]. (**D**) TSE vacuolation profiles in RIII mice ($n \geqslant 6$). All data shows mean ± SEM. Brain region areas: G1–9, grey matter scoring areas; G1, medulla; G2, cerebellum; G3, superior colliculus; G4, hypothalamus; G5, thalamus; G6, hippocampus; G7, septum; G8, retrosplenial and adjacent motor cortex; G9, cingulate and adjacent motor cortex. W1–W3, white matter scoring regions: W1, cerebellar white matter; W2, mesencephalic tegmentum; W3, cerebral peduncle. Dpi = days post inoculation.

(Fig. 1C). As reported previously, vacuolation profiles were similar in RIII mice across MV^R and MM^D (spleen and CNS) (Fig. 1D) with peaks in vacuolation intensity observed at medulla (G1), hypothalamus (G4) and septum (G7) regions (Bishop *et al.*, 2013). The MM^D (CNS) transmission resulted in TSE vacuolation profiles in RIII and C57BL6 mice that are similar to that of typical 129MM CNS transmissions. There was slight variation between the TSE vacuolation profiles of the MM^D (CNS) and MM^D (spleen) in the VM mice which could indicate changes in titre. MV^R resembled that of typical vCJD in 129MM individuals in RIII mice and MM^D showed typical profiles in RIII and VM mice. No TSE vacuolation profile was possible in VM or C57BL6 mice from MV^R because of lack of TSE vacuolation.

Immunohistochemical analysis was carried out to assess the amount and distribution of abnormal PrP deposition in each mouse line inoculated with spleen homogenate from the MV^R and spleen and brain homogenate from the MM^D. Variability in the amount of abnormal PrP deposition within experimental cohorts was apparent, but there appeared to be no clear differences in PrP distribution between the isolates inoculated into the same mouse line. In RIII mice, there was widespread PrP deposition of a granular nature in the hypothalamus and thalamus with a distinctive pattern of intense deposition of the CA2 region of the hippocampus (Fig. 2A, D, G and J) typical of vCJD in this mouse line. C57BL6 mice shared a similar deposition pattern to that of RIII mice with the distinctive CA2 targeting of the hippocampus (Fig. 2B, E, H and K). VM mice exhibited widespread fine punctate PrP deposition with pericellular deposits particularly in the thalamus, hypothalamus, midbrain and hippocampus (Fig. 2C, F and I). Occasional PrP plaques were observed in the corpus callosum (Fig. 2L) and are a typical feature of vCJD in VM mice.

When compared to previously-published 129MM vCJD transmissions associated with blood transfusion (Bishop *et al.*, 2008), there were a number of differences apparent in the MV^R and MM^D. These can be summarized as differences in attack rates (clinical signs and TSE vacuolation), increases in incubation periods and changes in TSE vacuolation profiles. The differences in attack rates between spleen and CNS transmissions is thought to be as a result of differences in titre, with peripheral organs such as the spleen having lower titres. However, the differences between the MV^R and MM^D spleen transmissions particularly in the VM mice could also be due to the modification of strain characteristics due to different host *PRNP* codon 129 genotype between MM^D and MV^R

Mouse subpassage of the MV and MM isolates defined strain characteristics

Inconsistences are not unusual in primary passage to mice due to crossing the species barrier and further passage is required to establish if such differences truly represent strain differences. Because of the variation observed upon primary passage of the MV^R and MM^D isolates, mouse-to-mouse subpassages of the MV^R and MM^D (CNS and spleen) were undertaken in order to fully characterize the prion strain of the isolates. In addition, we undertook subpassage of the MM^{D2008} and associated MM^{R2008} and the five isolates were then compared.

Subpassage from *Prnp*^{a} mice

A RIII or C57BL6 mouse which exhibited clinical signs and had positive TSE pathology was inoculated into the panel of wild-type mice and the strain characteristics analysed as above. Subpassage of the MV^R and MM^D (CNS and spleen) from both RIII and C57BL6 mice resulted in ~100% susceptibility in both clinical signs and TSE vacuolation. Incubation period rankings were of the order RIII < C57BL6 < VM (identical those of the MM^{D2008} and MM^{R2008}; however, there was a significant decrease in incubation time of the VM mice from the C57BL6 passage of the MM^D (CNS) compared to MV^R ($P < 0.001$) and MM^{D2008} ($P < 0.01$) (Fig. 3).

TSE vacuolation profiles were consistent between experimental cohorts from the five isolates with vacuolation particularly noted in the hypothalamus (G4) and septum (G7) of RIII and C57BL6 mice (Fig. 4A, B, G and H). There was minor variation apparent in the C57BL6 to C57BL6 passage from the MV^R where a peak in vacuolation intensity could be observed in the thalamus (G5) (Fig. 4H, indicated by an arrow). In VM mice, peaks in vacuolation intensity were in the medulla (G1), thalamus (G5) and septum (G7) (Fig. 4C and I). However, the C57BL6 to VM passage from the MM^D (CNS) (Fig. 4I) had only two peaks in vacuolation intensity in the medulla (G1) and thalamus (G5). There is minor variation in the MV^R when compared to the MM transmissions which could indicate that the strain has not yet stabilized, differences were also noted for the MM^D (CNS).

There was a widespread distribution of abnormal PrP deposition throughout the brain in all isolate/mouse line combinations predominantly of a diffuse nature (Fig. 5A, E and I). This was observed to be more intense in the thalamus, medulla and hippocampus where the CA2 region was targeted. PrP plaques were also present in all mouse lines/isolate combinations and were most commonly present in the cerebellum and corpus callosum but to a lesser degree in the MV^R subpassage.

Subpassage from *Prnp*^{b} mice

A VM mouse from each isolate exhibiting both clinical signs and TSE pathology was inoculated into the wild-type panel of mice and the strain characteristics analysed. Subpassage of MM^D (CNS and spleen) from VM mice gave between 87 and 100% attack rates (clinical signs and TSE vacuolation) to each mouse line whereas the subpassage from the MV^R in VM mice exhibited lower attack rates. These lower attack rates were particularly

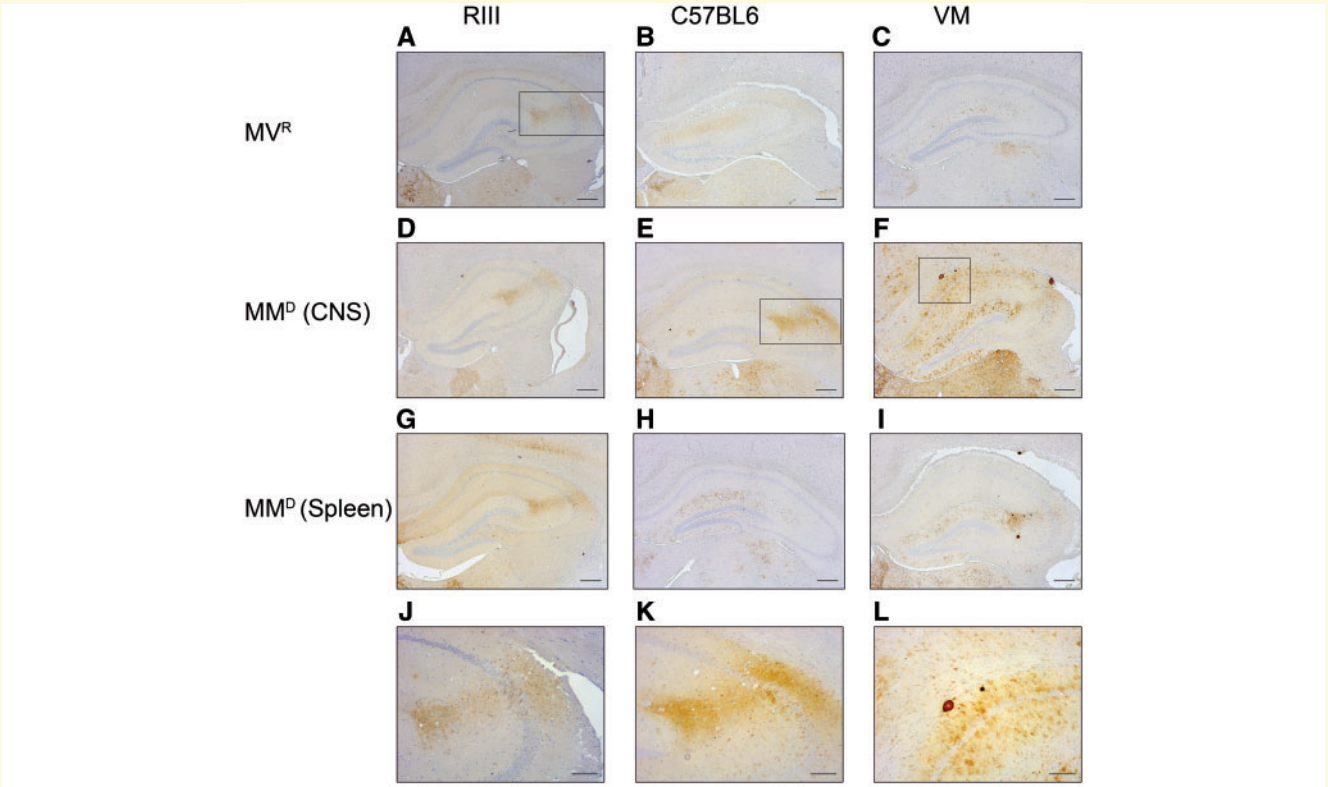

Figure 2 Abnormal PrP deposition in wild-type mice challenged with brain and spleen homogenates from two cases of vCJD. The two cases comprised an asymptomatic MV blood recipient (MV^R) and the MM donor to that individual (MM^D CNS and spleen). (**A**) RIII, (**B**) C57BL6, and (**C**) VM, mice challenged with MV^R. (**D**) RIII, (**E**) C57BL6, and (**F**) VM, mice challenged with MM^D (CNS). (**G**) RIII, (**H**) C57BL6, and (**I**) VM, mice challenged with MM^D (Spleen). (**J**) Abnormal PrP deposition in CA2 region of hippocampus from RIII mouse (inset region of **A**). (**K**) Abnormal PrP deposition in CA2 region of hippocampus from C57BL6 mouse (inset region of **E**). (**L**) PrP plaque in the corpus callosum of a VM mouse (inset region of **F**). Scale bars = 200 μm (**A**–**I**); 100 μm (**J**–**L**).

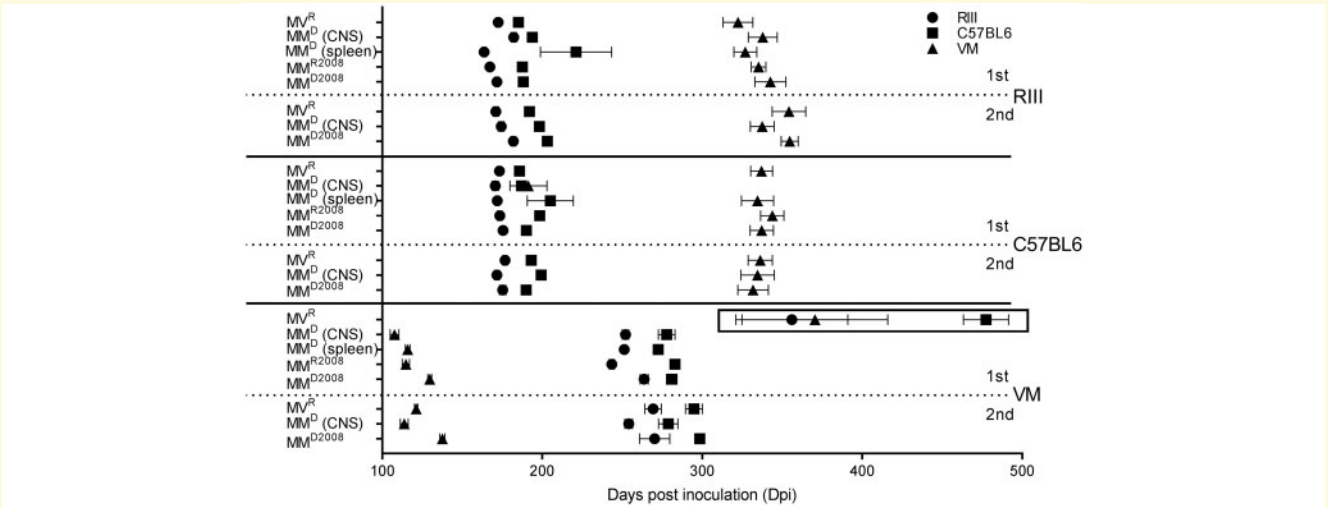

Figure 3 Incubation periods in wild-type (RIII, C57BL6 and VM) mice from the first and second mouse subpassage of brain and spleen homogenates from four cases of vCJD. The four cases comprise: an asymptomatic MV blood recipient (MV^R), the MM donor to that individual (MM^D), an MM blood donor (MM^{D2008}) and the associated MM blood recipient (MM^{R2008}). Incubation periods were calculated in mice showing clinical and pathological signs of TSE disease. Black box indicates significant changes in incubation periods ($P < 0.05$). All data shows mean ± SEM. Dpi = days post inoculation.

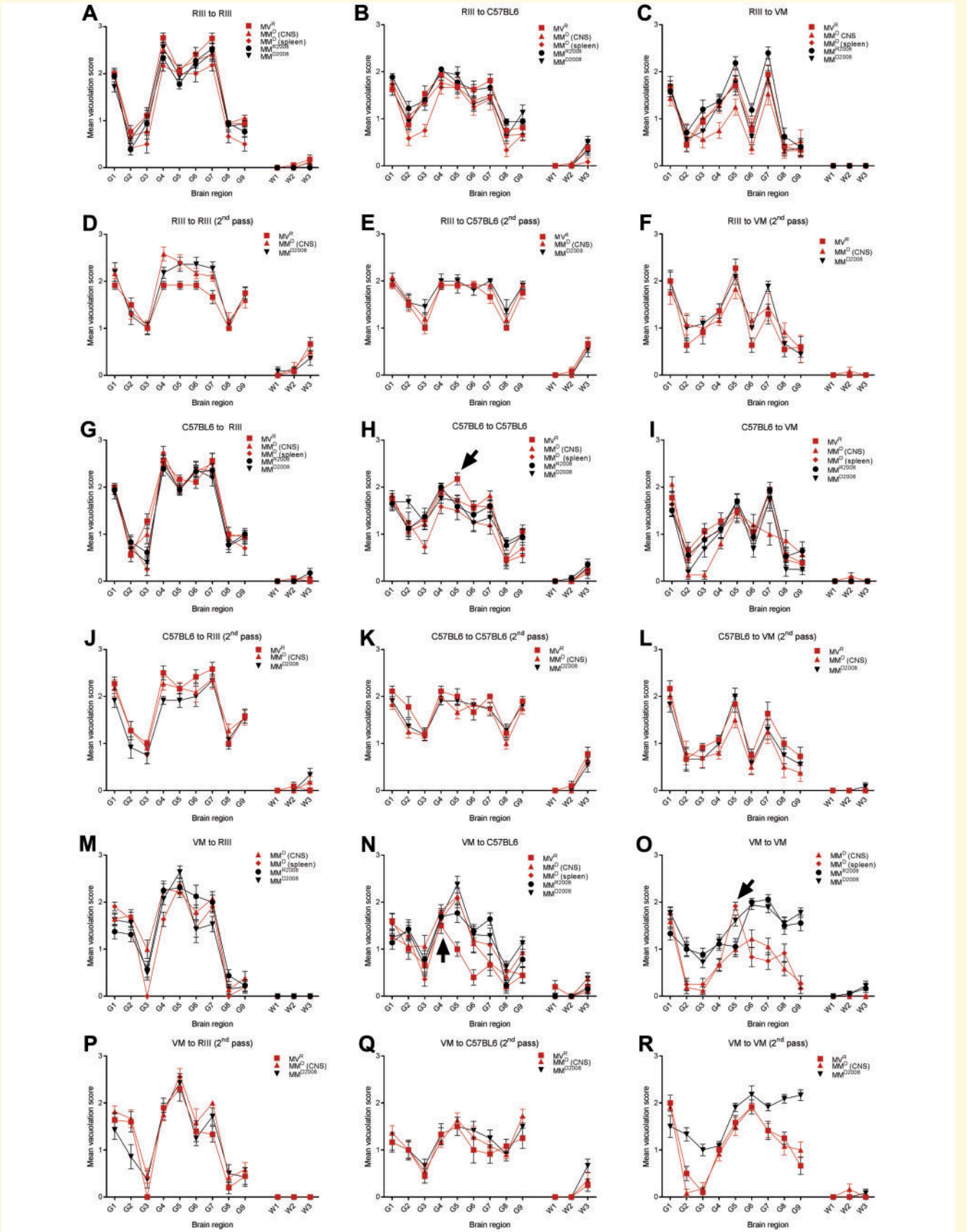

Figure 4 TSE vacuolation profiles in wild-type mice from the first and second mouse subpassage of brain and spleen homogenates from four cases of vCJD. The four cases comprise: an asymptomatic MV blood recipient (MV^R), the MM donor to that individual (MM^D), an MM blood donor (MM^{D2008}) and the associated MM blood recipient (MM^{R2008}). (**A**–**C**) First mouse subpassage from RIII mice. (**D**–**F**)

(continued)

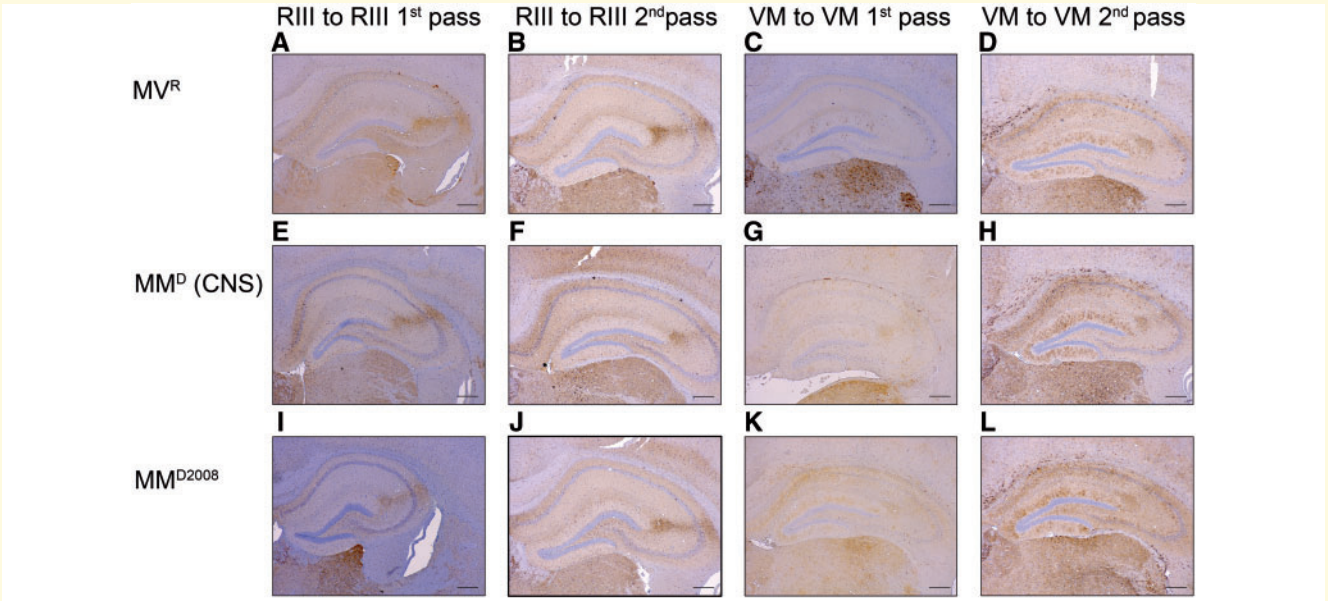

Figure 5 Abnormal PrP deposition in wild-type mice from the first and second mouse subpassage of brain and spleen homogenate from three cases of vCJD. The three cases comprise: an asymptomatic MV blood recipient (MV^R), the MM donor to that individual (MM^D CNS) and an MM blood donor (MM^{D2008}). (**A**) RIII mice from the first and (**B**) second subpassage of MVR. (**C**) VM mice from the first and (**D**) second subpassage of MV^R (**E**) RIII mice from the first and (**F**) second subpassage of MM^D (CNS). (**G**) VM mice from the first and (**H**) second subpassage of MM^D (CNS). (**I**) RIII mice from the first and (**J**) second subpassage of MM^{D2008}. (**K**) VM mice from the first and (**L**) second subpassage of MM^{D20008} Scale bars = 200 μm (**A**–**I**).

evident in the RIII line (18% clinical, 24% TSE vacuolation positive) and VM line (33% clinical, 60% TSE vacuolation positive). Incubation period rankings were of the order RIII < C57BL6 < VM from all isolates except the MV recipient. The ranking from the MV^R was of the order RIII < VM < C57BL6 with a significant increase in incubation period in RIII mice when compared to MM^D (spleen) ($P < 0.05$) and MM^{R2008} ($P < 0.001$), in C57BL6 mice when compared to MM^D (CNS and spleen), MM^{R2008} and MM^{D2008} ($P < 0.01$) and in VM mice when compared to MM^D (CNS and spleen) ($P < 0.01$) and MM^{R2008} ($P < 0.01$) (Fig. 3, indicated by black box). All MM transmissions were consistent in their attack rates and incubation period rankings regardless of whether they originated from CNS or spleen tissue. In contrast the MV^R had lower attack rates and a different incubation period ranking.

TSE vacuolation in RIII and C57BL6 mice from the MM^D (CNS and spleen) and C57BL6 mice from the MV^R was evident in the thalamus (G5) and septum (G7) (Fig. 4M and N). The vacuolation profile of the MV^R in the VM to C57BL6 passage showed a peak in vacuolation in the hypothalamus (G4) which was different to the other isolates that had a peak in the thalamus (G5) (Fig. 4N, indicated by arrow). There were insufficient mice from the MV^R subpassage to carry out a vacuolation profile in RIII and VM mice. The MM^D (CNS) VM to VM subpassage resulted in a similar profile albeit at lower levels of vacuolation to the MM^{D2008} and MM^{R2008}. The MM^D (spleen) exhibited higher levels of vacuolation in the thalamus (G5) rather than hippocampus (G6) of VM mice as was in the case in the other isolates (Fig. 4O, indicated by arrow). The MM CNS transmissions all demonstrated consistent TSE vacuolation profiles; however, the MM^D (spleen) showed minor variation in VM mice. The MV^R had differences in the VM to C57BL6 passage however most striking was the lack of TSE vacuolation positive RIII and VM mice.

Abnormal PrP accumulation was present in the mice with diffuse and punctate accumulation observed throughout the

Figure 4 Continued

Second mouse subpassage from RIII mice. (**G**–**I**) First mouse subpassage from C57BL6 mice. (**J**–**L**) Second mouse subpassage from C57BL6 mice. (**M**–**O**) First mouse subpassage from VM mice. (**P**–**R**) Second mouse subpassage from VM mice. Arrows indicates changes from typical vacuolation profiles. All vacuolation profiles were calculated from $n \geqslant 6$, data show mean ± SEM. Brain region areas: G1–9, grey matter scoring areas; G1, medulla; G2, cerebellum; G3, superior colliculus; G4, hypothalamus; G5, thalamus; G6, hippocampus; G7, septum; G8, retrosplenial and adjacent motor cortex; G9, cingulate and adjacent motor cortex. W1–W3, white matter scoring regions: W1, cerebellar white matter; W2, mesencephalic tegmentum; W3, cerebral peduncle.

brain particularly in the hippocampus and thalamus with PrP plaques present on occasion in the corpus callosum and cerebellum (Fig. 5C, G and K).

When compared to the typical MM subpassages, MM^{D2008} and MM^{R2008}, the MM^{D} and MV^{R} both demonstrated differences in strain characteristics at the first mouse-to-mouse passage. Unexpectedly, we observed differences in the MM^{D} (CNS) subpassage in incubation periods and rankings and TSE vacuolation profiles whereas the MM^{D} (spleen) was more consistent with the typical MM transmissions from CNS material. Of importance was that differences were observed in the MV^{R} subpassage in incubation periods and rankings and TSE vacuolation profiles. These changes in strain characteristics are unlikely to be due to source material (spleen) as we did not observe similar changes in the MM^{D} spleen and could be ascribed to genotype of this individual.

Strain properties of the asymptomatic MV individual are identical to those of codon 129MM individuals

As variation was still observed upon the first mouse-to-mouse subpassage, a second mouse-to-mouse subpassage was performed from the MV^{R}, MM^{D} (CNS) and the MM^{D2008}. These isolates were chosen as a number of variations had been observed in the MV^{R} and MM^{D} (CNS) subpassages, the MM^{D2008} was chosen as an example of a typical vCJD subpassage The stabilization of a prion strain can require multiple passages in the same host. Once stabilized the incubation period and neuropathological characteristics will remain constant if they are continually propagated under constant conditions, i.e. PrP genotype of the mice (Bruce, 2003).

Subpassage from *Prnp*^{a} mice

In contrast to the first mouse-to-mouse subpassage, the second mouse-to-mouse passage of the MV^{R} and MM^{D} (CNS) displayed ~100% susceptibility in clinical signs and TSE vacuolation across all experimental cohorts as did the MM^{D2008}. Incubation period rankings were in the order RIII < C57BL < VM for isolates passaged through RIII and C57BL6 mice as observed in the first subpassage of the MM^{D2008}. The incubation period of VM mice of the MM^{D} (CNS) in the C57BL6 subpassage increased compared to the first subpassage thus giving a similar incubation period for the MM^{D2008} and MV^{R} (Fig. 3). TSE vacuolation profiles and PrP deposition patterns showed no differences between the three isolates (Figs 4D–F, J–L and 5B, F and J). Biochemical analysis of PrP^{res} from the RIII to RIII subpassage showed the same glycoprofile between MV^{R}, MM^{D} (CNS) and MM^{D2008} with a dominant di-glycosylated fragment of ~30 kDa and an unglycosylated band of 20 kDa similar to that of the type 2B glycoprofile observed from vCJD human brain tissue (Fig. 6A, Supplementary 1). These results indicate that the same strain of prion agent has been isolated from the MV^{R}, MM^{D} (CNS) and MM^{D2008}.

Subpassage from *Prnp*^{b} mice

The second subpassage through VM mice of the MV^{R} resulted in a change of the ranking observed in the first subpassage (RIII < VM < C57BL6); with all three isolates now demonstrating the ranking VM < RIII < C57BL6 (Fig. 3). TSE vacuolation profiles of the three isolates in the VM to RIII and VM to C57BL6 subpassage followed the same vacuolation trend in each mouse line and these were near identical to the profiles resulting from the MM^{D2008} in the first subpassage (Fig. 4P and Q). PrP deposition patterns were similar to that of RIII and C57BL6 mice with no differences between the MV^{R} and the two MM blood donors.

In the VM to VM subpassage, the MM^{D2008} exhibited a greater intensity of TSE vacuolation overall; however, it followed the same distribution pattern of vacuolation with peaks in the medulla (G1) and hippocampus (G6). As in the first subpassage the MM^{D2008} exhibited greater vacuolation intensity in the septum (G7), retrosplenial cortex (G8) and cingulate and motor cortex (G9) than the MM^{D} (CNS) (Fig. 4R). There were no differences in PrP deposition patterns (Fig. 5D, H and L). Upon VM to VM subpassage there were no differences upon biochemical analysis of the MV^{R}, MM^{D} (CNS) and MM^{D2008} isolates with a dominant di-glycosylated fragment of ~30 kDa, mono-unglycosylated band of ~27 kDa and an unglycosylated fragment at 20 kDa (Fig. 6B, Supplementary 1).

Overall analysis of the strain properties of each isolate indicates that two stable strains have emerged; one strain

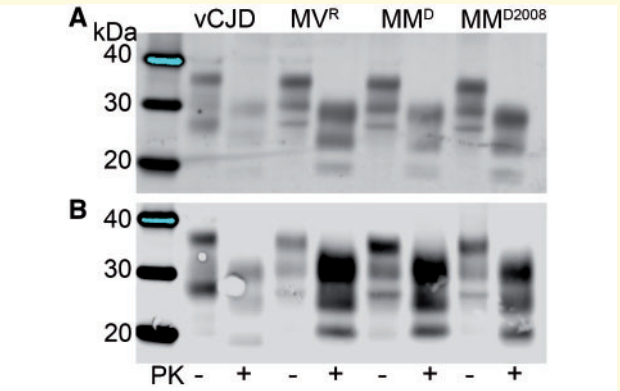

Figure 6 Western blot analysis of brain extracts from the second mouse subpassage of RIII (A) and VM (B) wild-type mice challenged with three cases of vCJD. The three cases comprised: an asymptomatic MV blood recipient (MV^{R}), the MM donor to that individual [MM^{D} (CNS) and an MM blood donor (MM^{D2008})]. A human vCJD standard positive control is shown for reference with the typical abnormal prion protein (PrP^{res}) type 2B. All samples were treated with proteinase K (indicated by plus symbol). The anti-prion protein detection antibody used was 6H4. Molecular weight markers are shown in kDa. PK = Proteinase K.

passaged through RIII and C57BL6 (*Prnp*^{a}) mice and one strain through VM (*Prnp*^{b}) mice. These mouse-adapted strains are consistent with those identified previously in both 129MM vCJD and BSE (301C and 301V) transmission studies (Bruce *et al.*, 2002; Ritchie *et al.*, 2009). The data presented here demonstrate that vCJD in an asymptomatic MV individual is of an identical strain to that identified in clinical vCJD individuals of the codon 129MM genotype. It also indicates human-to-human transmission through different host genotypes has not caused any strain adaptation and that the BSE/vCJD has remained constant.

Discussion

This study provides the first evidence that codon 129 genotype of the host does not influence the strain characteristics of vCJD. In light of the identification of a clinical MV case of vCJD and abnormal PrP deposition in appendixes of all codon 129 genotypes, this finding is important for public health. Strain properties are often influenced by the *PRNP* genetic background of the host and modifications in the strain properties can result in different host ranges and clinical outcomes; until now, it was unknown whether a different genetic background could alter vCJD characteristics. The characterization of the infectious agent from spleen from this asymptomatic case of vCJD in a codon 129 heterozygous individual has provided answers to these key questions.

Our study finds no evidence of a novel vCJD strain after passage through an MV individual; however, this could be because of the methionine-carrying prion proteins acting as a dominant species. While other studies have demonstrated that replication of the infectious agent is possible in all three genotypes, the 129VV genotype was clearly less susceptible to disease (Bishop *et al.*, 2006). Thus the presence of methionine in the 129MV genotype may dominate any replication process giving rise to strain characteristics identical to that in 129MM genotypes. At present, we cannot be sure of the effect of valine homozygosity on strain characteristics although mouse studies indicate there may be potential for different strain characteristics to emerge (Takeuchi *et al.*, 2013; Fernandez-Borges *et al.*, 2017).

Our initial study showed that the spleen of the MV recipient was infectious (Bishop *et al.*, 2013) but on this primary passage there were inconsistencies in strain characteristics when compared with those of previously characterised vCJD cases from MM individuals. The BSE/vCJD strain has consistently produced unique characteristics upon primary passage to mouse strain typing panels. Most notably these include high attack rates, a characteristic TSE vacuolation profile in RIII mice and a consistent incubation period ranking; RIII followed by C57BL6 ~100 days later than VM mice (Bruce *et al.*, 1994, 1997; Ritchie *et al.*, 2009; Diack *et al.*, 2012, 2017). The majority of prion strains tend to show low attack rates with long incubation periods upon primary passage. Further mouse subpassages generally show a shortening in incubation periods and ~100% susceptibility in terms of clinical and pathological signs of disease indicative of the removal of the species barrier. These properties then remain consistent throughout further subpassages (Bruce, 2003). As the studies in the MV recipient demonstrated inconsistences at primary passage, a full strain characterization study was required. However, the strain characteristics stabilized and were identical to that of previous vCJD and BSE transmissions with the second mouse subpassage resulting in either a 301C or 301V type TSE strain as determined by mouse line (Bruce *et al.*, 2002; Ritchie *et al.*, 2009).

The inconsistences noted at primary passage in attack rate and lack of TSE vacuolation, could have been a consequence of the interaction between human and mouse genetic backgrounds i.e. *PRNP* codon 129 genotype and *Prnp*^{a} or *Prnp*^{b} or could be due to a lower titre of infectivity in the spleen isolates causing longer incubation periods and decreased numbers of mice exhibiting signs of prion disease within their lifespan (Bruce *et al.*, 2001; Bruce, 2003; Ritchie *et al.*, 2009).

Similar to earlier studies of extraneural tissue transmission, we find that the strain of agent is not altered by the tissue of origin (Bruce *et al.*, 2001; Ritchie *et al.*, 2009). *In vivo* transmission studies have suggested that different prion strains can be isolated from CNS and lymphoid tissue in the same host the mechanisms of which are not yet understood (Beringue *et al.*, 2012). Beringue *et al.* (2012) also demonstrated that prions can exist in the periphery for nearly one-third of a host's life before CNS detection. In vCJD a body of evidence including the transmission of vCJD through blood transfusion (Peden *et al.*, 2004; Hewitt *et al.*, 2006; Wroe *et al.*, 2006; Gillies *et al.*, 2009), detection of PrP^{TSE} through PMCA (Bougard *et al.*, 2016) and *in vivo* infectivity studies have shown that prion infectivity can be present without CNS involvement (Bishop *et al.*, 2013). Three retrospective studies in anonymized UK appendix samples have revealed the presence of abnormal PrP in all three codon 129 genotypes (Ironside *et al.*, 2000; Gill *et al.*, 2013; Public Health England, 2016). This has given a prevalence estimate of 1 in 2000 individuals in the UK with abnormal PrP in their appendix that could be considered asymptomatic vCJD. It is currently not known exactly what the abnormal PrP present in these appendix samples represents. Possibilities include infection with vCJD, infection with another prion disease or artefact unrelated to prion disease. The results from this study of an asymptomatic individual may be important in deciphering the results from these retrospective appendix studies.

We have demonstrated that vCJD strain properties have not been impacted by host genotype, human-to-human transmission through blood transfusion, tissue of origin or point of infectivity in the disease course. However, with the identification of a primary case of clinical vCJD in a 129 heterozygous individual and abnormal PrP in

appendixes of all codon 129 genotypes it is clear that continued human surveillance is required to identify new cases of vCJD and recognize any differences in disease phenotype that could be indicative of changes to the prion strain. Strain characterization studies of the clinical case of primary vCJD in a codon 129 heterozygous individual are currently being undertaken. Any changes in prion strain could lead to changes in infectious properties, which is an immediate concern for public health and it is essential that we do not become complacent in our approach to these fatal diseases.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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