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Agent strain variation in human prion disease: insights from a molecular and pathological review of the National Institutes of Health series of experimentally transmitted disease

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Six clinico-pathological phenotypes of sporadic Creutzfeldt–Jakob disease have been characterized which correlate at the molecular level with the type (1 or 2) of the abnormal prion protein, PrP^{TSE}, present in the brain and with the genotype of polymorphic (methionine or valine) codon 129 of the prion protein gene. However, to what extent these phenotypes with their corresponding molecular combinations (i.e. MM1, MM2, VV1 etc.) encipher distinct prion strains upon transmission remains uncertain. We studied the PrP^{TSE} type and the prion protein gene in archival brain tissues from the National Institutes of Health series of transmitted Creutzfeldt–Jakob disease and kuru cases, and characterized the molecular and pathological phenotype in the affected non-human primates, including squirrel, spider, capuchin and African green monkeys. We found that the transmission properties of prions from the common sporadic Creutzfeldt–Jakob disease MM1 phenotype are homogeneous and significantly differ from those of sporadic Creutzfeldt–Jakob disease VV2 or MV2 prions. Animals injected with iatrogenic Creutzfeldt–Jakob disease MM1 and genetic Creutzfeldt–Jakob disease MM1 linked to the E200K mutation showed the same phenotypic features as those infected with sporadic Creutzfeldt–Jakob disease MM1 prions, whereas kuru most closely resembled the sporadic Creutzfeldt–Jakob disease VV2 or MV2 prion signature and neuropathology. The findings indicate that two distinct prion strains are linked to the three most common Creutzfeldt–Jakob disease clinico-pathological and molecular subtypes and kuru, and suggest that kuru may have originated from cannibalistic transmission of a sporadic Creutzfeldt–Jakob disease of the VV2 or MV2 subtype.

Keywords: prion diseases; neuropathology; neurodegenerative disorders; phenotype; strain typing

Abbreviations: CJD = Creutzfeldt–Jakob disease; NIH = National Institutes of Health; TSEs = transmissible spongiform encephalopathies

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Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases are invariably fatal neurodegenerative disorders affecting humans and other mammals such as sheep, deer, elk and cattle. In humans, TSEs occur worldwide as sporadic, genetic or acquired disease and comprise three major disease entities with variable though overlapping phenotypes: Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome and fatal insomnia. In addition, the term *kuru* has been attributed to an acquired human prion disease falling within the phenotypic spectrum of CJD that affected primarily the Fore linguistic group of the Eastern Highlands of Papua New Guinea.

In TSE, an abnormal, beta-sheet rich and partially protease-resistant isoform (PrP^{TSE}) of the cellular prion protein, PrP^{C}, accumulates in the nervous system, and to a lesser extent in other organs, and represents the hallmark of the disease (Brown *et al*., 1986; Roberts *et al*., 1986). The conversion of PrP^{C} to PrP^{TSE} is a post-translational event, and involves a conformational change of the protein (Caughey *et al*., 1991; Pan *et al*., 1993) that can be transmitted by an autocatalytic mechanism (Bieschke *et al*., 2004). PrP^{TSE} is thought to be an essential, if not the exclusive, component of the transmissible agent, or prion (Prusiner, 1998; Weissmann, 2004; Aguzzi *et al*., 2008).

Prion diseases comprise a broad spectrum of clinico-pathological phenotypes that show heterogeneity in disease duration, symptomatology and distribution of brain lesions such as spongiosis, neuronal loss and gliosis, as well as presence and morphology of amyloid plaques (Gambetti *et al*., 2003; Ghetti *et al*., 2003; Kretzschmar and Parchi, 2007). Different prion strains are believed to be the main cause of TSE phenotypic diversity (Aguzzi *et al*., 2007). TSE strains were originally defined by their distinct disease phenotypes upon transmission to syngenic animals, which persist on serial transmission (Fraser *et al*., 1973; Bruce *et al*., 1991). Within a given host species, prion strains differ mainly in their incubation times, the distribution of central nervous system vacuolation that they produce and whether or not they induce amyloid plaques. In addition, the host genotype variability in the gene encoding PrP^{C} (*PRNP*), as determined by polymorphisms or mutations, has also been recognized as a causal factor for phenotypic heterogeneity (Bruce *et al*., 1991; Palmer *et al*., 1991; Goldfarb *et al*., 1992; Goldman *et al*., 1994; Barron *et al*., 2001; Gambetti *et al*., 2003; Ghetti *et al*., 2003). Distinct human strains of the prion agent have been demonstrated after experimental transmission (Telling *et al*., 1996; Bruce *et al*., 1997; Hill *et al*., 1997; Lasmezas *et al*., 2001; Korth *et al*., 2003; Nonno *et al*., 2005; Bishop *et al*., 2006; Kobayashi *et al*., 2010) but remain to be fully characterized.

Uncertainties also remain regarding the molecular basis of TSE strains and the relationship between the agent strains and PrP (Aguzzi *et al*., 2007). Distinct PrP^{TSE} profiles or types have been found in both humans and animals and can be distinguished by specific physicochemical properties such as size after protease treatment, degree of protease resistance, and conformational stability and glycoform ratio (Kascsak *et al*., 1986; Bessen and Marsh, 1994; Monari *et al*., 1994; Collinge *et al*., 1996; Parchi *et al*., 1996, 1997, 2000; Somerville *et al*., 1997; Safar *et al*., 1998; Notari *et al*., 2004; Baron *et al*., 2007; Wemheuer *et al*., 2009). The different PrP^{TSE} types can be associated with distinct disease phenotypes even in subjects with the same *PRNP* genotype (Parchi *et al*., 1999, 2000). Furthermore, it has been shown that properties of the PrP^{TSE} type related to protein conformation, such as the size of the protease-resistant core, are often maintained after inter-species transmission (Telling *et al*, 1996), although changes may also occur in certain recipient genotypes (Hill *et al*., 1997; Kobayashi *et al*., 2010). In summary, circumstantial evidence suggests that the strain phenotypes are enciphered in distinct tertiary or even quaternary structures of PrP^{TSE}, although the formal proof is still absent. Given these uncertainties and to avoid confusion, throughout this manuscript the term *prion strain* is used to refer to the property of the prion agent to induce a specific disease phenotype in a given host genotype after transmission, whereas the term PrP^{TSE} type refers to only the biochemical PrP^{TSE} properties, usually defined by western blotting, that have been associated with a given disease phenotype.

Two major human PrP^{TSE} types are known, which have been extensively reproduced among laboratories: 'type 1' with a relative molecular mass of the protease-resistant core of 21 kDa and the primary cleavage site at residue 82, and 'type 2' with a relative molecular mass of 19 kDa and the primary cleavage site at residue 97 (Parchi *et al*., 1996, 1997, 2000).

PrP^{TSE} types 1 and 2, in conjunction with the genotype at the methionine (M) / valine (V) *PRNP* codon 129, largely correlate with phenotypic variability in human sporadic TSEs and provide a molecular basis for disease classification (i.e. MM1, MM2, VV1, etc.) (Parchi *et al*., 1996, 1999). Furthermore, the same human PrP^{TSE} types detected in sporadic TSEs were also found to be associated with distinct phenotypes in iatrogenic CJD, kuru and familial CJD (Parchi *et al*., 1997, 2000), thus raising the possibility that the same prion strains would be isolated upon transmission from all these TSE forms, independent of their apparently different aetiology.

The experimental transmission of CJD and kuru was first accomplished in the 1960s through the pioneering studies of Gajdusek, Gibbs and Alpers at the National Institutes of Health (NIH) (Gajdusek *et al*., 1966; Gibbs *et al*., 1968). This ground-breaking discovery led to an intensive 30-year research programme during which several hundreds of cases of various neurological disorders were inoculated in non-human primates. Clinical, neuropathological and biological data concerning these transmissions have been published but have not included studies to identify distinct phenotypes by means of lesion profiling or molecular characterization of PrP^{TSE} by western blotting, PrP immunohistochemistry and paraffin embedded tissue (PET) blotting. We address here the issues of prion strain variation in human TSEs and their molecular basis, with a systematic re-analysis of disease characteristics in matched human and non-human primate tissues obtained from the NIH series of transmitted cases, which provides a historically unique resource to study the issue of human prion strains in a comprehensive series of transmissions to primates.

Materials and methods

Case and tissue selection

Human cases

A total of 99 human cases were selected from the NIH database of transmitted TSEs based on the availability of frozen tissue. In addition, 13 transmitted kuru cases were selected despite the lack of frozen tissue. According to the original clinico-pathological and genetic analyses obtained from the NIH database and previous publications (Beck *et al*., 1973; Scrimgeour *et al*., 1983; Brown *et al*., 1986, 1994; Goldfarb *et al*., 1991; Cervenáková *et al*., 1998), the selected cases consisted of 90 sporadic CJD, 5 familial CJD carrying the E200K mutation coupled with methionine at codon 129 in both mutated and wild-type alleles (E200K-129MM), 2 iatrogenic CJD resulting from contaminated stereotactic intracerebral EEG needles (Bernoulli *et al*., 1977) and 15 kuru cases. All cases had a neuropathologically verified spongiform encephalopathy, and in 99 cases, the diagnosis was confirmed by western blotting (all but 13 kuru cases).

Clinical analysis

Clinical data for the human TSE cases were available in all cases, and to a large extent included in a previous publication (Brown *et al*., 1994).

Incubation times from inoculation to the onset of clinical symptoms and the duration of symptoms for the non-human primates were drawn from NIH charts and previously published data (Brown *et al*., 1994). For these analyses, we considered animals that were inoculated intracerebrally with a 5–20% phosphate buffered saline suspension of fresh-frozen brain tissue. We applied this criterion with the aim to exclude the least number of animals, since it was previously found (Brown *et al*., 1994) in serial dilution experiments that the length of incubation time does not change significantly in the 10 or 100 fold dilution range, while it is significantly prolonged at a 10^5 dilution. Furthermore, it should be considered that most of the animals included in the analyses were exposed to a 10% homogenate, and the relative proportion of animals injected with a 5, 10 or 20% brain homogenate among the most significant groups of animals was very similar (e.g. 5, 77 and 18%, respectively, for the group of squirrel monkeys injected with sporadic CJD MM1 and 17, 71 and 12%, respectively, for the squirrel monkeys injected with sporadic CJD VV2).

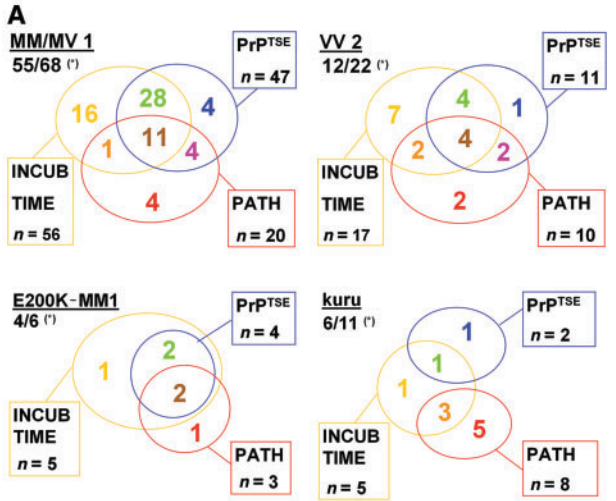
Besides the concentration, the volume of the inoculated homogenate also varied, but this mainly accorded with the size (species) of primate: chimpanzees (not included in the present study), for example, routinely received 0.1 ml, whereas squirrel monkeys usually received 0.05 ml (P. Brown, personal communication). Overall, a total of 130 transmission experiments were considered for the calculation of incubation times.

Primate brain tissues

After screening the NIH animal databases, all positive (i.e. neuropathologically verified spongiform encephalopathy) animals that had been inoculated with one of the selected human TSE cases, and for which tissues (either frozen or fixed or both) were still available, were sampled. Tissue from four different non-human primate species (squirrel, capuchin, spider and African green monkeys) was obtained. To include as many animals as possible, sampling was performed irrespective of the availability of the information on incubation time. The relationship between numbers of human cases analysed and the number of animals available for the analyses of incubation time, histopathological features and PrP^{TSE} typing for the most significant groups (i.e. squirrel monkeys at first passage) is provided in Fig. 1A. The large majority of tissues were obtained from animals injected at first passage; however, fixed tissue was also taken from a group of squirrel monkeys that were injected either at second passage or after serial passages (Fig. 1B).

Overall, frozen tissue from 100 primates was available for PrP^{TSE} typing analyses, and formalin fixed tissue from 94 animals was obtained for neuropathological examination. Frozen brain tissue was obtained from squirrel ($n = 72$), capuchin ($n = 17$), spider ($n = 8$) and African green ($n = 3$) monkeys. Large tissue blocks were available in most cases (i.e. ~95%), whereas small tissue fragments, usually from the cerebral cortex, were obtained from a few brains. Tissue samples for western blot analyses were taken from the cerebral cortex (usually frontal cortex) in most cases; samples from the cerebellum and striatum were additionally obtained in six spider monkey brains.

Fixed brain tissue was obtained from squirrel ($n = 68$, 50 at first passage and 18 at second or serial passage), spider ($n = 11$), capuchin ($n = 10$) and African green ($n = 5$) monkeys. The tissue consisted either of formalin fixed, paraffin embedded blocks or formalin fixed brain slices, or both. For each case, depending on availability, sections were obtained from as many as possible of the following brain regions: frontal, temporal, parietal and occipital cortices, hippocampus, entorhinal cortex, striatum, thalamus, hypothalamus, midbrain and cerebellum.



B

	2dP	sP
sCJDMM1	2/2	3/3
kuru	1/2	4/11

Figure 1 (**A**) Relationship between number of animals available for the analyses of incubation time (INCUB TIME), histopathological features (PATH), and PrP^{TSE} typing (PrP^{TSE}) for the largest groups of human TSE subtypes transmitted to squirrel monkeys (first passage). Asterisk indicates the number of different inocula/number of examined animals. (**B**) Number of squirrel monkeys examined histopathologically after second passage (2dP) or serial passage (sP). *n*/*n* = number of different inocula/number of examined animals.

Neuropathology

Semiquantitative evaluation of spongiform changes was carried out using haematoxylin and eosin stained sections. Eleven brain regions were selected for examination (listed above). Spongiform change was scored on a 0–3 scale (not detectable/mild/moderate/severe) and lesion profiles were obtained. The lesion profile is a well-established semiquantitative method of measuring the targeting of spongiform changes to different brain regions and reliably discriminates between TSE strains in mice and other species and between sporadic CJD subtypes in humans (Bruce *et al*., 1996, 1997; Parchi *et al*., 1999).

PrP immunohistochemistry and PET blot were performed successfully only on paraffin embedded tissue blocks (the long-standing formalin-fixed tissues yielded negative results, presumably due to the very long storage in formalin). Tissue from 30 squirrel monkeys (24 at first passage and 6 at second or serial passage) was examined with these two techniques. The 24 animals of the first group were injected with homogenates from: sporadic CJD MM1 ($n = 6$), sporadic CJD VV2 ($n = 5$), sporadic CJD MM with a 20 kDa PrP^{Sc} core ($n = 1$) (see 'Results' section), sporadic CJD MV2 with kuru plaques (sporadic CJD MV 2K) ($n = 2$), sporadic CJD MV1 ($n = 2$), kuru ($n = 3$), iatrogenic CJD MM1 ($n = 2$) and familial CJD E200K–129 M ($n = 3$). The 6 animals of the second group were injected with homogenates from sporadic CJD MM1 ($n = 3$) and kuru ($n = 3$). Staining was obtained in all sections that were available for the histopathological analysis. Paraffin sections from formalin-fixed blocks were processed using the monoclonal antibodies 3F4 with epitope at PrP residues 108–111 at 20 μg/ml concentration (Signet Labs, MA, USA) according to previously published protocols (Parchi *et al*., 1996). PrP deposits were classified according to their morphology (i.e. synaptic, granular, focal plaque-like). PET blot was performed according to a previously described protocol (Schulz-Schaeffer *et al*., 2000). Examination of the staining patterns for both PrP immunohistochemistry and PET blot were performed blind to the results of the molecular and histopathological analysis and the type of human inoculum.

Protein analysis

Preparation of samples including PrP^{TSE} purification, western blotting and PrP^{TSE} typing were performed according to established methods (Notari *et al*., 2004, 2007; Parchi *et al*., 2000, 2009*a*). In particular, all samples were homogenized in lysis buffer plus [100 mmol/l NaCl, 10 mmol/l EDTA, 0.5% (v/v) Nonidet P 40, 0.5% (w/v) sodium deoxycholate, 100 mmol/l Tris–HCl, pH 6.9] and digested with proteinase K (Roche Diagnostics, specific activity by certificate of analysis: 47.9 U/mg) at a final concentration of 5 U/ml. Purified PrP^{TSE} was obtained from about 400 mg of tissue from the cerebral cortex, which was subjected to three cycles of sarkosyl extraction and differential centrifugation to yield the P3 pellet. Running gels with different separating discriminatory power (i.e. 5, 6.5 and 15 cm in length) were used. All samples but those from capuchin monkeys were probed with the monoclonal antibody 3F4 (residues 106–110, Signet Labs, MA, USA) (Kascsak *et al*., 1986; Zou *et al*., 2010), whereas capuchin monkey homogenates, whose PrP is not recognized by 3F4, were probed with the monoclonal antibody 9A2 (residues 99–101). The immunoreactivity was visualized by enhanced chemiluminescence (ECL standard or ECL Plus, GE Healthcare) on Kodak BioMax Light films (Eastman Kodak Co.) and with LAS-3000 camera (Fujifilm). To quantify the PrP^{TSE} content, we compared the signal intensity by densitometry (Aida Image Analyzer v.4.15 software, Raytest) with a standard curve, obtained by loading in each gel a serial dilution ($n = 4$) of a sporadic CJD sample chosen as standard.

Molecular genetic analysis

All human samples were re-analysed, whereas the *PRNP* sequence of non-human primates was obtained from previously published results (Cervenáková *et al*., 1994). In this regard it is noteworthy that at variance with humans, all non-human primate species carry methionine at codon 129 (Cervenáková *et al*., 1994). The open reading frame of the *PRNP* was amplified as previously described, using DNA purified from brain tissue (Parchi *et al*., 1996). The polymerase chain reaction product was visualized on a 1% agarose gel to detect potential insertion mutations or deletions. Potential point mutations were initially revealed by analyses by denaturing high-performance liquid chromatography and subsequently confirmed by direct sequencing of *PRNP* open reading frame. Finally, the codon 129 genotype was examined by digestion with the restriction endonuclease Nsp 1.

Results

Molecular typing of human inocula

Molecular classification of sporadic, genetic and iatrogenic CJD and of two kuru cases was performed according to PrP^{TSE} type (Parchi *et al*., 1996, 1997, 1999) and *PRNP* genotype. In addition, information regarding the codon 129 genotype was available for 6 out of the 13 kuru cases lacking frozen material. Among them, 4 were VV, 1 MV and 1 MM. Overall, the immunoblot analysis of PrP^{TSE} from the human samples was consistent with previously published data. All but one sample fitted within the spectrum of human PrP^{TSE} profiles previously described in sporadic CJD (Parchi *et al*., 1996, 1997; Notari *et al*., 2004), classic iatrogenic CJD with MM genotype (Parchi *et al*., 1997), familial CJD (Monari *et al*., 1994; Parchi *et al*., 2000) and kuru (Parchi *et al*., 1997). In particular, the two kuru cases with frozen tissue were VV and MV at codon 129 and showed PrP^{TSE} type 2, as we previously reported (Parchi *et al*., 1997), whereas, as expected from previous results (Notari *et al*., 2004; Parchi *et al*., 2009*b*), most cases belonging to the MV 2K sporadic CJD subtype showed a duplet including a 20 kDa band in addition to the 19 kDa fragment (Fig. 2). The only atypical case was MM at codon 129 and showed a proteinase K-resistant PrP^{TSE} core of about 20 kDa, which migrated slightly faster than typical type 1 (Fig. 2).

The demographics and classification of the different human TSE subgroups, based on the molecular analyses, are shown in Table 1. In addition to the kuru cases, the series comprises other historically relevant cases reported in the literature before the advent of molecular biology and the discovery of PrP^{TSE} and *PRNP*. They include one case classified as ataxic CJD by Brownell and Oppenheimer (1965), the ataxic CJD case described by Gomori *et al*. (1973) and the atypical CJD case, with widespread kuru plaques, reported by Schoene *et al*. (1981). Molecular analyses showed that both cases with the ataxic sporadic CJD variant were VV2, whereas the case from Schoene *et al*. (1981) was genotyped as MM at codon 129 and corresponds to the atypical case described above with the 20 kDa PrP^{TSE} band (Fig. 2).

This patient had worked as a neurosurgeon for many years, which raises the theoretical possibility that he had an iatrogenic form of CJD, although there was no record of him participating in an operative procedure on a CJD patient (Schoene *et al*. 1981).

Incubation times

Incubation times in monkeys varied significantly among the different CJD subgroups (Table 2).

Notably, incubation times in both squirrel and spider monkeys were shorter in animals inoculated with tissue from sporadic CJD MM1 than in those inoculated with sporadic CJD VV2 or kuru, although this was statistically significant only for the VV2 group in squirrel monkeys (Table 2). Furthermore, familial CJD (E200K-MM1) transmitted the disease to squirrel monkeys with incubation times comparable to sporadic CJD MM1.

Neuropathology and lesion profiles

Among the triad of lesions that characterize the neuropathology of human TSEs (i.e. spongiform change, neuronal loss and gliosis), only spongiform change was prominent in the non-human primates. None of the animals showed amyloid plaques.

Squirrel monkeys

Neuropathological examination revealed that each of the examined animals had features of, and could be easily assigned to, either one of two clearly distinct pathological phenotypes (henceforth referred to as A and B). The histopathological features were highly consistent among individual animals. Phenotypes A and B showed significant differences in the characteristics of the spongiform change, the lesion profile and the pattern of PrP deposition (Table 3). In phenotype A the spongiform change consisted of relatively small, delicate vacuoles that affected mainly the cerebral cortex, the caudate and putamen nuclei and the thalamus, whereas the hippocampus, the hypothalamus, the brainstem and the cerebellum were relatively spared (Figs 3 and 4). In addition, there was a distinctive, focal, laminar distribution of spongiform change in the fifth layer of the occipital cortex (Fig. 3), whereas spongiform change affected all cortical layers with an inconstant predominant involvement of the deep layers in the other lobes (data not shown). Finally, PrP immunohistochemistry and PET blotting revealed a delicate diffuse synaptic pattern of PrP^{TSE} deposition in all areas showing spongiform changes (Figs 3 and 5).

In contrast to phenotype A, spongiform changes in phenotype B consisted of larger vacuoles (Fig. 3), showed a more clear-cut laminar pattern in the deeper layers of the cerebral cortex, and affected predominantly subcortical structures rather than the cerebral cortex (Fig. 4, Table 3). Immunohistochemistry and PET blotting revealed focal, relatively small, plaque-like PrP deposits in addition to the synaptic staining (Figs 3 and 5).

The analysis of a group of 18 animals in which CJD or kuru prions were serially transmitted (Fig. 1B) either in squirrel monkeys (2 days passage) or through other primate species such as chimpanzees or spider monkeys before the re-injection in squirrel monkeys (serial passages) also showed two distinct phenotypes corresponding to phenotype A and phenotype B based on histopathological and immunohistochemical examination of the cerebral cortex, which was available for all cases.

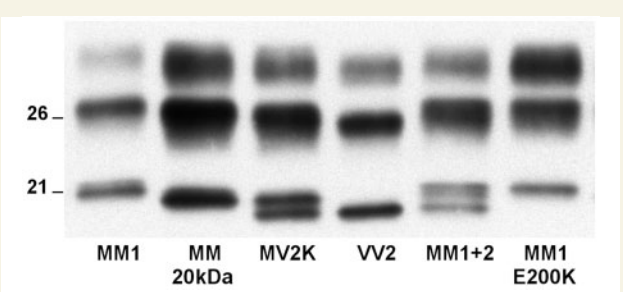


Figure 2 Western blot profiles of PrP^{TSE} extracted from human brains of the NIH series of transmitted disease (Brown *et al*., 1994). A case for each of the different molecular subtypes that were inoculated in monkeys is shown (lanes 1–6). Lane 2 shows the atypical MM case with a 20 kDa PrP^{TSE} core. Brain homogenates were treated with proteinase K and probed with 3F4. Approximate molecular masses are in kilodaltons.

Other primate species

Although the material available was not sufficient for a detailed comparison of lesion profiles between groups of animals receiving different inocula, neuropathological features of the disease in capuchin, spider and African green monkeys were consistent with those observed in squirrel monkeys.

In particular, two phenotypes reproducing distinctive features of phenotype A and B in squirrels, such as vacuole size, intracortical distribution of spongiform changes and the pattern of PrP deposition, were seen in both capuchin and spider monkeys,

Table 1 Demographic characteristics and classification of transmitted cases

PRNP	MM + MV	MM	MM	MM	MV	VV	E200K-M	MM, VV, MV MM	
PrP^{TSE} type	1	1 + 2	20 kDa[a]	2	2	2	1	ND/2	1
Form	sCJD	sCJD	sCJD	sCJD	sCJD	sCJD	fCJD	kuru	iCJD
Number of cases	66 + 3	4	1	1	2	13	5	15	2
Age at onset (years)	61.1 (40–78)	61.7 (42–89)	54	49	53 (51–55)	60.4 (41–70)	59.2 (55–62)	22.8 (10–45)	21 (19–23)
Duration (months)	4.3 (1–24)	6.1 (1.5–15)	15	15	17 (16–18)	6.2 (3.5–12)	4.6 (3–6.5)	11.2 (6–17)	8[b]

sCJD = sporadic CJD; fCJD = familial CJD; iCJD = iatrogenic CJD; ND = not determined in MM cases.
a PrP^{TSE} showed a relative molecular size of 20 kDa, thus intermediate between type 1 and type 2.
b Disease duration available for only one case.

Table 2 Incubation times (in days) in different monkey species

TSE subtype[a]	Squirrel Mean ± SD inocula/animals	Spider Mean ± SD inocula/animals	Capuchin Mean ± SD inocula/animals	African green Mean ± SD inocula/animals
sCJD MM/MV 1	755 ± 108 49/56	852 ± 87 6/6	1387 ± 316 13/13	1497 ± 126 6/6
sCJD VV2	884 ± 94* 11/17	1147 ± 308 4/4	–	–
fCJD E200K-MM1	651 ± 73 3/5	–	–	–
Kuru	828 ± 94 5/5	913 ± 262 7/9	1477 ± 269 4/4	–
iCJD MM1	618 ± 131 2/5	–	–	–

In each species the same TSE case has been used for five inoculations at most. sCJD = sporadic CJD; fCJD = familial CJD; iCJD = iatrogenic CJD.
a For each species only the groups with at least four animals are shown.
*$P < 0.05$ versus sporadic CJD MM1.

Table 3 Main features of pathological phenotypes A and B in monkeys

	Phenotype A	Phenotype B
Vacuole size	Small	Large
Intracortical spongiosis	All layers affected in fronto-temporal and parietal cortices. Laminar distribution in the fifth layer of the occipital cortex	Laminar pattern with predominant involvement of deeper layers
Lesion profile	Cortico-striato-thalamic pattern. Sparing of brainstem	Most subcortical regions are more affected than the cerebral cortex
PrP staining	Punctate, fine (synaptic)	Synaptic + focal small plaque-like deposits

whereas in the small group of African green monkeys examined (which were all inoculated with sporadic CJD MM1), all animals showed features consistent with phenotype A.

Biochemical characterization of PrP^{TSE} in non-human primates

Compared with humans, the unglycosylated protease-resistant core of PrP^{TSE} in monkeys always migrated in the 20–21 kDa range and was therefore never cleaved close enough to the C-terminus to reach the lower relative molecular mass (i.e. 19 kDa) of human PrP^{TSE} type 2. In this regard it is noteworthy that, at variance with humans, all non-human primate species carry methionine at codon 129 (Cervenáková *et al*., 1994).

Nevertheless, two significantly different migration PrP^{TSE} core profiles (henceforth referred to as 'a' and 'b') could be distinguished in both squirrel (Fig. 6) and capuchin monkeys (Fig. 7), particularly when long gels with increased resolution were used. The first (more common) profile showed an unglycosylated fragment of the same size as the human PrP^{TSE} type 1, whereas the second profile included a faster migrating fragment of about 20 kDa in addition to the 21 kDa band (Figs 6 and 7). Profile 'a' was seen in both squirrel and capuchin monkeys with the pathological phenotype A, while profile 'b' was linked to phenotype B in these two species. At variance with squirrel and capuchin monkeys, the protease-resistant PrP^{TSE} core from spider monkeys had a molecular size of about 21 kDa (profile 'a') in all animals analysed, irrespective of whether they had a pathological phenotype A or B. Finally, PrP^{TSE} extracted from African green monkeys, which was examined only in cases with phenotype A, also showed a relative molecular mass of ~21 kDa consistent with profile 'a'.

In addition to its size, the relative amount of the three major PrP^{TSE} bands that are seen after immunoblotting (the so-called 'glycoform ratio') often characterizes TSE subtypes or prion strains. PrP^{TSE} glycoform ratio showed a significant heterogeneity in both squirrel and spider monkeys. Two PrP^{TSE} profiles with a statistically significant difference in glycoform ratio were found that correlated with pathological phenotype A and B, respectively, in both squirrel and spider monkeys; in addition, in squirrel monkeys, the two glycopatterns also matched the two profiles 'a' and 'b' based on PrP^{TSE} size. The more common profile was characterized by the dominance of the diglycosylated fragment of PrP^{TSE} (or by similar amounts of monoglycosylated and diglycosylated PrP^{TSE} forms in spiders), whereas the second profile showed a larger amount of the monoglycosylated fragment (Fig. 8). The difference between the two PrP^{TSE} glycoform patterns was especially pronounced in spider monkeys (Fig. 8B). Finally, in capuchin monkeys, the glycoform ratio of PrP^{TSE} did not show a significant difference between the migration profile 'a' and 'b'. However, at variance with squirrel monkeys, the number of cases analysed, especially for profile 'b', was very low.

We have calculated the total amount of PrP^{TSE} in each brain homogenate obtained from the squirrel monkey cerebral cortex and found a significant variability among the affected animals, without any significant correlation with the disease phenotype (Fig. 9A). To search for possible differences in other biochemical properties such as the solubility in detergents, we have also purified PrP^{TSE} in a selected group of squirrel monkeys showing either PrP^{TSE} migration profile 'a' ($n = 8$) or profile 'b' ($n = 4$) and evaluated the relative proportion of PrP^{TSE} in the detergent-soluble (S3) and insoluble (P3) fractions. The amount of PrP^{TSE} in the P3 fraction was very similar between the two groups, whereas squirrels affected by phenotype A had a significantly higher amount of

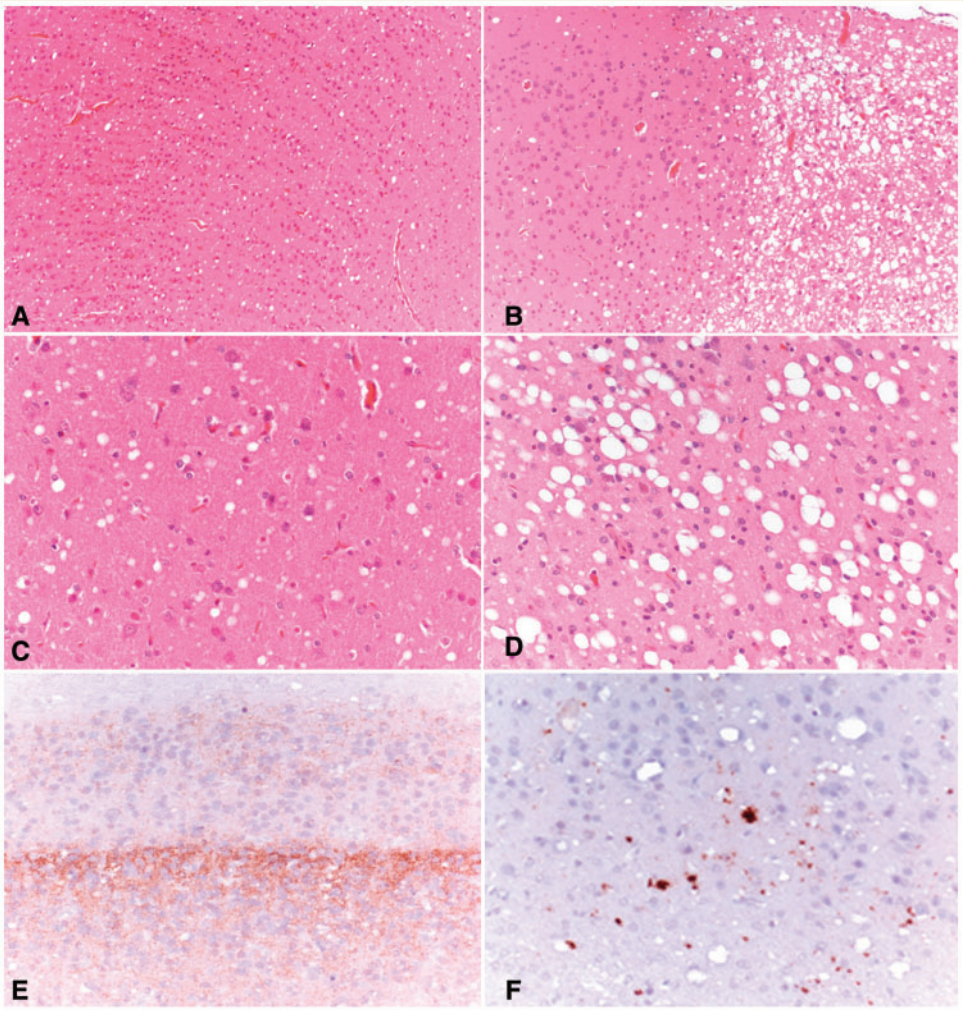


Figure 3 Histopathological features of phenotype A (**A**, **C**, **E**; animal inoculated with sporadic CJD MM1) and phenotype B (**B**, **D**, **F**; animal inoculated with sporadic CJD VV2) in squirrel monkeys. (**A** and **C**) Spongiform changes consisting of relatively small delicate vacuoles in the occipital cortex. Haematoxylin and eosin 100× (**A**), 200× (**C**). (**B** and **D**) Spongiform changes consisting of relatively large vacuoles in the entorhinal cortex (**B**) and thalamus (**D**). Haematoxylin and eosin 100× (**B**), 200× (**D**). (**E**) Synaptic PrP staining in the occipital cortex, immunohistochemistry (IHC) with monoclonal antibody 3F4, 200×; (**F**) focal, plaque-like immunoreactivity in the occipital cortex, IHC with monoclonal antibody 3F4 200×.

'detergent soluble' PrP^{TSE} compared with those with phenotype B (Fig. 9B).

Pathological phenotypes, PrP^{TSE} profiles and the type of human inocula

Both the pathological phenotype and PrP^{TSE} profile (either the electrophoretic mobility or the glycopattern or both) in the monkeys were significantly related to the type of inocula. The data were largely consistent across all monkey species, although the data from squirrel monkeys were much more informative due to the higher numbers of samples. The details concerning the correlation between type of inoculum, pathological phenotypes and PrP^{TSE} profile, as well as the number of animals analysed for each group, are reported in Table 4. Overall, samples from sporadic CJD MM1 and MV1, iatrogenic CJD MM1, sporadic CJD MM1+2 and familial CJD carrying the E200K mutation coupled with methionine at codon 129 produced phenotype A and PrP^{TSE} profile 'a', whereas the homogenates from sporadic CJD VV2, sporadic CJD MV 2K and from the kuru cases produced phenotype B and PrP^{TSE} profile 'b'. Exceptions to this rule were spider monkeys, which showed a PrP^{TSE} protease-resistant fragment corresponding to profile 'a' in all animals analysed, irrespective of whether they had a pathological phenotype A or B. Nevertheless, the correlation between the PrP^{TSE} glycopattern and the pathological phenotypes A and B in spider monkeys was striking (Fig. 8, Table 4). As in squirrel monkeys, the most common glycoform profile was observed in animals with pathological phenotype A, while the second profile, characterized by a dominant monoglycosylated form and a striking

under-representation of the diglycosylated form, was linked to phenotype B.

Finally, a single sporadic CJD MM 2C, in which only PrP^{TSE} type 2 was demonstrated in two samples from the cerebral cortex and cerebellum, also showed phenotype A and PrP^{TSE} profile 'a', whereas the atypical MM case with the 20 kDa PrP^{TSE} core showed phenotype B and PrP^{TSE} profile 'b'.

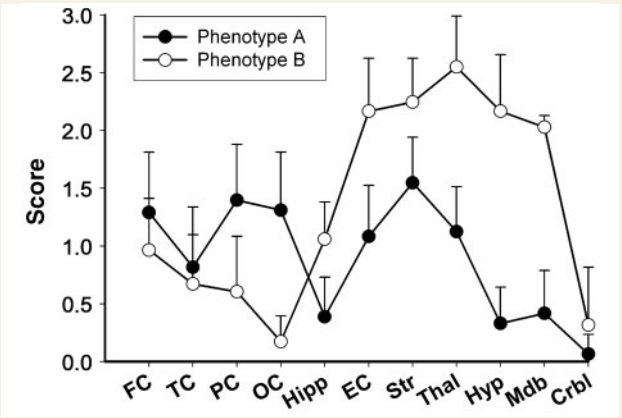


Figure 4 Regional profile of spongiform changes in squirrel monkeys inoculated with different CJD subtypes or kuru. Two distinct profiles can be identified: in phenotype A, a significant spongiform change is seen in only the cerebral cortex, striatum and, to some extent, the thalamus, whereas in phenotype B, the spongiform change predominates in subcortical regions with a relative sparing of the cerebral cortex. FC = frontal cortex; TC = temporal cortex; PC = parietal cortex; OC = occipital cortex; Hipp = hippocampus; EC = entorhinal cortex; Str = striatum; Thal = thalamus; Hyp = hypothalamus; Mdb = midbrain; Crbl = cerebellum.

Discussion

In the present study we have compared the transmission properties of the most common phenotypic subtypes of sporadic, familial and iatrogenic CJD as well as kuru in different non-human primate species. This series of transmitted cases is historically unique, as it is based on the large NIH study that provided the essential evidence that human TSEs are transmissible.

The transmission data in primates strongly suggest that two distinct prion strains are linked to the three most common sporadic CJD variants, which account for the great majority of human prion diseases. More specifically, our results show that the myoclonic CJD phenotype associated with PrP^{TSE} type 1 in codon 129 MM or MV *PRNP* genotypes (Parchi *et al*., 1999) is related to a prion strain that is distinct from that associated with the ataxic and the kuru-plaque phenotypes linked to PrP^{TSE} type 2 and VV or MV, respectively (Parchi *et al*., 1999).

The results of this study also show that kuru prions have transmission properties equivalent to sporadic CJD VV2 and MV2 with kuru plaques (MV 2K). The finding is in line with previous results underlying the striking similarities in both clinico-pathological features and PrP^{TSE} properties between sporadic CJD VV2 or MV 2K and kuru (Hainfellner *et al*., 1997; Parchi *et al*., 1997; McLean *et al*., 1998; Brandner *et al*., 2008; McLean, 2008). Overall, the findings confirm the idea that kuru originated from the chance consumption and cannibalistic diffusion of tissues from an individual with sporadic CJD, and point to the probability of either a VV2 or an MV 2K case, since we did not find evidence

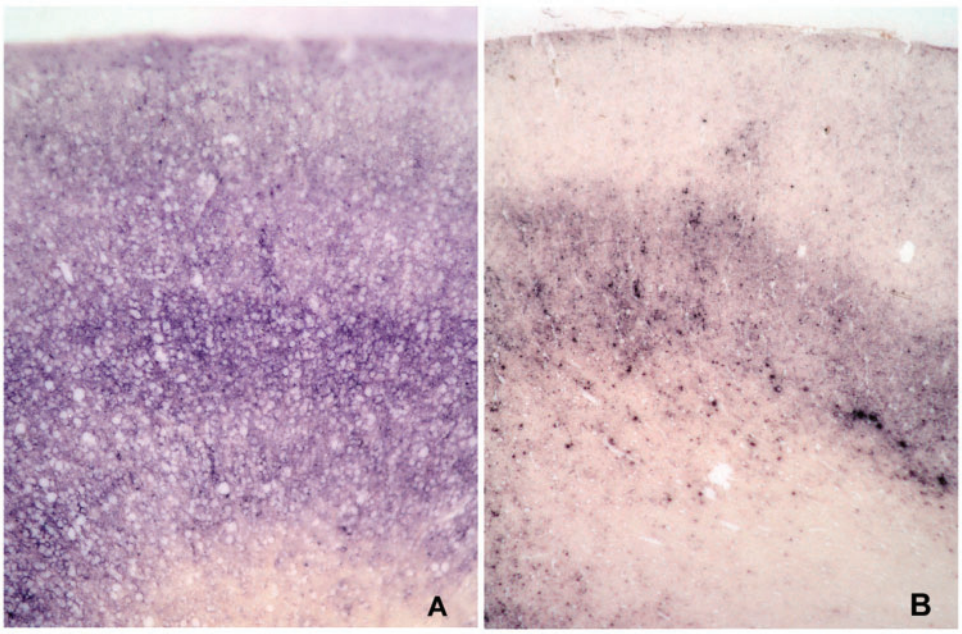


Figure 5 PET blot features in squirrel monkeys with phenotype A (**A**) and B (**B**). (**A**) Diffuse 'synaptic' PrP staining in the frontal cortex. (**B**) Combined synaptic and focal, plaque-like PrP immunoreactivity in the deep layers of the frontal cortex.

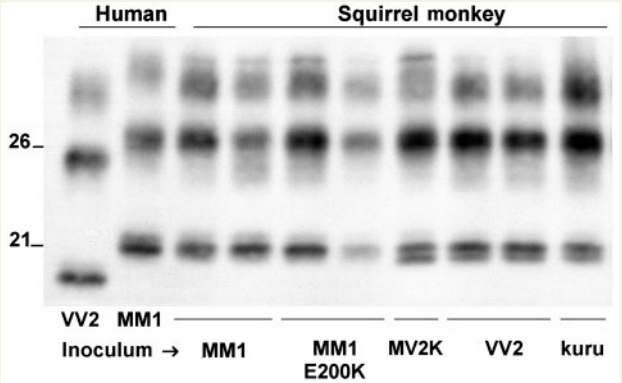


Figure 6 Immunoblot analysis of protease-resistant PrP^{TSE} extracted from brains of squirrel monkeys (lanes 3–10) inoculated with different CJD subtypes or kuru. Brain homogenates were treated with proteinase K and probed with 3F4. Approximate molecular masses are in kilodaltons.

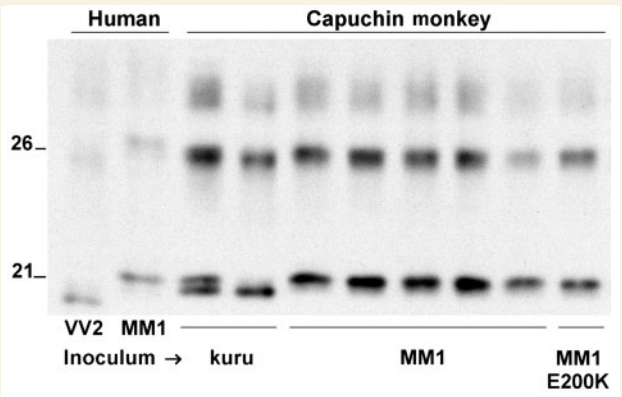
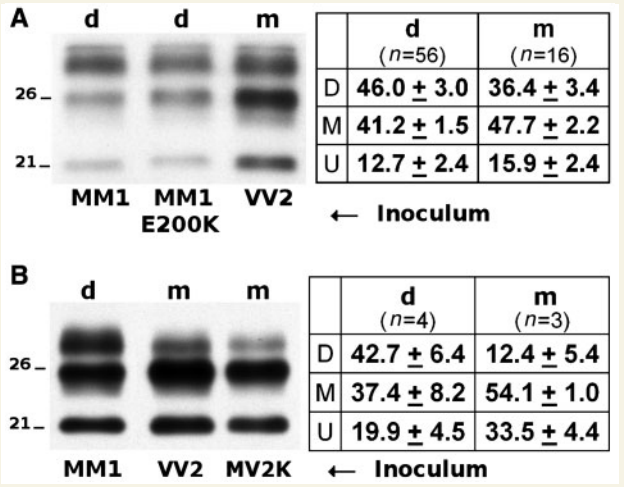


Figure 7 Immunoblot analysis of protease-resistant PrP^{TSE} extracted from brains of capuchin monkeys (lanes 3–10) inoculated with different CJD subtypes or kuru. Brain homogenates were treated with proteinase K and probed with monoclonal antibody 9A2. Approximate molecular masses are in kilodaltons.



	d (*n*=56)	m (*n*=16)
D	46.0 ± 3.0	36.4 ± 3.4
M	41.2 ± 1.5	47.7 ± 2.2
U	12.7 ± 2.4	15.9 ± 2.4

	d (*n*=4)	m (*n*=3)
D	42.7 ± 6.4	12.4 ± 5.4
M	37.4 ± 8.2	54.1 ± 1.0
U	19.9 ± 4.5	33.5 ± 4.4

Figure 8 Analysis of PrP^{TSE} glycoform ratio in squirrel (**A**) and spider (**B**) monkeys inoculated with different CJD subtypes or kuru. Two distinct PrP^{TSE} glycopatterns, 'd' and 'm' are shown. Pattern 'd' is characterized by a predominant diglycosylated PrP^{TSE} form, while in pattern 'm' the monoglycosylated form is the most abundant. Brain homogenates were treated with proteinase K and probed with monoclonal antibody 3F4. Approximate molecular masses are in kilodaltons.

of sporadic CJD MM1 prions in experimentally transmitted kuru, even in subjects carrying the MM or MV genotype. Interestingly, recently performed 'trace back' experiments have indicated that transmission of sporadic CJD VV2 to a 129 MM individual through a dura implant was also the mechanism generating the iatrogenic CJD phenotype associated with plaques, called p-dCJD (Kobayashi *et al*., 2010). These findings are consistent with our observations in the kuru case with MM genotype and in the atypical MM case with kuru plaques and the PrP^{TSE} 20 kDa band. Thus, it appears that this prion strain, although more frequently associated with the V codon 129 allele, also transmits to individuals homozygous for the codon 129 methionine allele, in which it is molecularly and phenotypically distinguishable from the most common MM/MV1-associated strain. Of note, iatrogenic CJD related to contaminated growth hormone injection is also characterized by amyloid plaque deposition of kuru type and also affects the MM genotype in addition to 129 VV and MV recipients (Billette de Villemeur, 1994; Will, 2003; Brown, 2006).

The different transmission properties of sporadic CJD type 1 inocula in MM or MV codon 129 genotype compared with the type 2 inocula in VV or MV genotype are also consistent with data recently obtained in transgenic mice and *in vitro* models. The studies conducted in transgenic mice expressing either human MM, VV or MV genotype are of particular interest for a comparison with our results, although most of them have focused on variant CJD and, to a lesser extent, on genetic TSEs, whereas the data collected thus far concerning the sporadic CJD subtypes and kuru are patchy and inconclusive. Sporadic CJD prions transmitted to transgenic mice with either MM or VV genotype, with incubation times that are consistent with our conclusion that the MM1/MV1 and VV2/MV 2K sporadic CJD subtypes are linked to two different human prion strains (Korth *et al*., 2003). Similar data on incubation time were obtained in another study where a few sporadic CJD inocula were transmitted to transgenic mice expressing human PrP with M at codon 129 to be compared with the transmission properties of bovine spongiform encephalopathy and variant CJD prions (Asante *et al*., 2002).

The interaction between host genotype of the 'substrate' and the genotype and PrP isoform of the prion 'seed' have also been recently modelled *in vitro* using the protein misfolding cyclical amplification (PMCA) technique. It has been found that seeds from MV1 amplified efficiently in MM (and to a lesser degree MV) substrate and therefore behave similarly to MM1, whereas MV2, similarly to VV2, amplified efficiently in VV substrate (Jones *et al*., 2008), which is also consistent with the results of the present study.

The transmission properties of kuru and CJD isolates have also been compared in transgenic and wild-type mice.

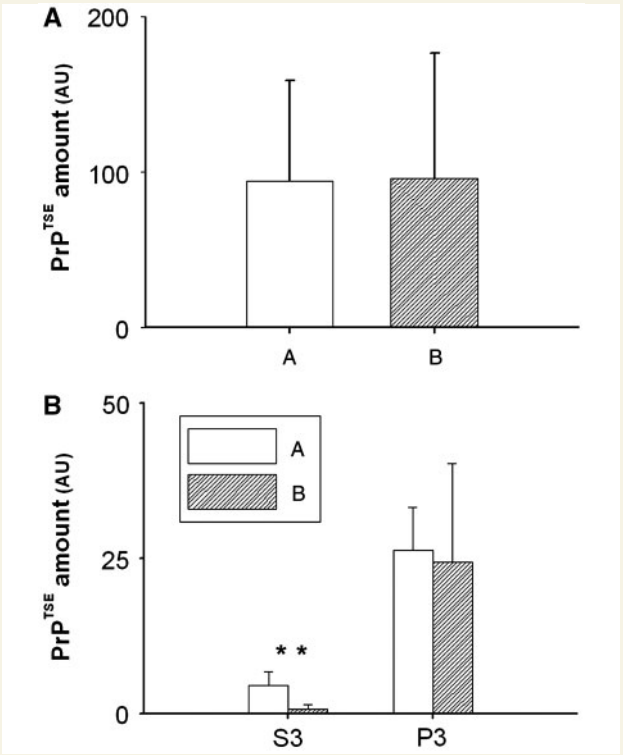


Figure 9 (**A**) Amount of proteinase K-resistant PrP^{TSE} in squirrel monkey total brain homogenates. The two groups of animals showing the PrP^{TSE} profile 'a' ($n = 56$) or 'b' ($n = 16$) on western blot are compared. Data are expressed as mean ± SD. (**B**) Amount of purified PrP^{TSE} (not treated with proteinase K) in detergent-soluble (S3) and detergent-insoluble (P3) fractions of squirrel monkey brain homogenates. Selected animals showing the PrP^{TSE} profile 'a' ($n = 8$) or 'b' ($n = 4$) on western blot are compared. In both panels the protein amount is expressed in arbitrary units (AU) and is calculated by densitometric analyses using a dilution curve of a sporadic CJD sample chosen as standard (see 'Materials and methods' section). a versus b, $^{**}P < 0.004$ (Mann–Whitney rank sum test).

Wadsworth *et al*. (2008) inferred that kuru prions are distinct from variant CJD and have transmission properties equivalent to those of classical (sporadic) CJD prions, whereas Manuelidis *et al*. (2009) argued that the kuru agent is a unique isolate distinct from CJD. Unfortunately, the fact that Wadsworth *et al*. (2008) failed to fully characterize the heterogeneity associated with sporadic CJD prions after transmission, and Manuelidis *et al*. (2009) did not mention which isolate of sporadic CJD (i.e. molecular/pathological subtype) was used for transmission, limits the interpretation of their results and the comparison with the present study. Nevertheless, we would agree with the general conclusion of Wadsworth *et al*. (2008) but also show that kuru prions do not match the entire spectrum of sporadic CJD.

An increasing body of data indicates the existence of CJD cases with PrP^{TSE} types 1 and 2 co-occurrence in the same brain (Parchi *et al*., 1999; Puoti *et al*., 1999). We recently found that in sporadic CJD the co-occurrence of PrP^{TSE} types 1 and 2 involves about 35% of sporadic CJD cases and especially affects subjects with a codon 129 MM genotype (Calì *et al*., 2009; Parchi *et al*., 2009*b*). In the present series of transmitted cases, the percentage of MM cases with the co-occurrence of PrP^{TSE} types 1 and 2 was significantly lower, which is consistent with the lack of sufficient tissue to perform regional analyses. Nevertheless, it is noteworthy that apart from the atypical MM case with the kuru plaques and the 20 kDa band and the MM kuru cases, all MM cases with features of the MM 2C sporadic CJD subtype showed identical transmission properties independent of the presence of PrP^{TSE} type 2 and its relative amount. This also included one case in which we were able to detect only PrP^{TSE} type 2 in the two samples that were available. There are two possible explanations for these results. Potentially, the primate species used were much more susceptible to MM1 than MM 2C prions and this prevented the appearance of histopathological features related to MM 2C replication. Alternatively, the MM 2C phenotype in humans is not related to a specific prion strain but rather to host genotypic factors that are responsible for the (co-)appearance of type 2 and the associated MM 2C phenotype. Further transmission

Table 4 Pathological phenotype (Path) and PrP^{TSE} migration pattern in different monkey species according to human inoculum

TSE subtype (inoculum)	Squirrel		Spider		Capuchin		African green	
	Path	PrP^{TSE}	Path	PrP^{TSE}	Path	PrP^{TSE}	Path	PrP^{TSE}
sCJDMM1 or MV1	A (16/20)	a (42/47)	A (3/4)	a^d (4/4)	A (4/4)	a (12/12)	A (4/4)	a (2/2)
sCJD MM 1+2	A (1/3)	a (3/3)	–	–	–	–	–	–
fCJD E200K-MM1	A (3/3)	a (2/4)	A (1/1)	a^d (1/1)	–	a (2/2)	A (1/1)	a (1/1)
iCJD MM1	A (1/2)	a (2/2)	–	–	A (2/2)	–	–	–
sCJD MM2	–	–	–	–	A (1/1)	a (1/1)	–	–
sCJDVV2	B (7/10)	b (8/11)	B (1/1)	a^m (2/2)	–	–	–	–
kuru	B (5/8)	b (2/2)	B (4/4)	–	B (3/3)	b (2/2)	–	–
sCJD MV2	B (2/2)	b (2/2)	B (1/1)	a^m (1/1)	–	–	–	–
CJD MM 20 kDa	B (2/2)	b (1/1)	–	–	–	–	–	–

(*n*/*n*)= number of inocula (TSE cases)/number of animals analysed. A and B refer to the two pathological phenotypes described in Table 3. a and b refer to the two PrP^{TSE} migration patterns shown in Figs 6 and 7. d and m refer to the two glycotypes shown and explained in Fig. 8. sCJD = sporadic CJD; fCJD = familial CJD; iCJD = iatrogenic CJD.

studies using animals that are less permissive to CJD MM/MV 1 or CJD VV2 prions will be needed to definitively solve this issue.

The present data also have implications for the understanding of familial and acquired forms of prion diseases. We previously showed that type 1 and type 2 PrP^{TSE} are present in all forms of CJD independent of the apparent aetiology of the disease, i.e. sporadic, inherited or acquired by infection (Parchi *et al*., 1997, 2000). These observations raise the critical question of whether the same basic strains present in sporadic CJD are also associated with the familial forms of the human disease and, if this is the case, whether the unique phenotypic features of some familial prion diseases can be explained by an effect of the *PRNP* mutation that is independent from the prion strain.

The present data on the transmission of iatrogenic CJD and familial CJD to primates along with results of transmission experiments of familial CJD prions (E200K-129 M and I210V-129 M haplotypes) in bank voles (Nonno *et al*., 2005) and familial CJD (E200K-129 M and E200K-129V) in transgenic humanized mice (Asante *et al*., 2009) are consistent with this hypothesis. Interestingly, in the latter study it was found that at variance with the E200K-129MM inoculum, the E200K-129VV inoculum transmitted with a prolonged incubation time and the affected mice showed plaque-like focal deposits, which parallels our findings with sporadic CJD MM1 and VV2 prions in non-human primates.

Previous studies on sporadic CJD MM1, the most common sporadic human CJD subtype, have raised the question of whether this relatively large group of cases is homogeneous and related to a single prion strain or rather represents a heterogeneous group including two or more sporadic CJD subtypes. The present study, based on the largest group of transmitted sporadic CJD MM1 cases to date, showed homogeneous results in terms of incubation time, type of spongiform changes, lesion profile and PrP^{TSE} properties. The collective results, therefore, strongly support the idea that a single prion strain is associated with sporadic CJD MM1, and are in keeping with our original classification, at variance with other classification systems that considered our MM1 cases as a heterogeneous group including two strain-related subtypes (Zanusso *et al*., 2001; Hill *et al*., 2003).

For many years it has been debated whether the study of fragment size and glycoform ratio of PrP^{TSE} provide sufficiently distinctive molecular markers to allow identification of prion strains. Our data indicate that in humans, the PrP^{TSE} profile provides significant information about the causative strain, although it is not indicative of it under all circumstances in the absence of histopathological data. For example, it is still difficult to distinguish between cases with the same PrP^{TSE} type (for example, between MM 2T and MM 2C) without knowing the histopathological data. Furthermore, recent work from our group has shown that in some instances the histopathological examination is even more sensitive than the biochemical PrP^{TSE} typing in the recognition of sporadic CJD cases with mixed phenotypic features (Parchi *et al*., 2009*b*).

Concerning the value of PrP^{TSE} typing in the recognition of strains affecting different species, it has been previously shown that both PrP^{TSE} size and glycoform pattern may change after inter-species transmission (Hill *et al*., 1997; Kobayashi *et al*., 2010). In agreement with this view, transmission of sporadic CJD MM/MV 1 to non-human primates consistently reproduced the size but not the glycoform ratio of the original type. Even more strikingly, transmission of sporadic CJD VV2 or MV 2K did not reproduce the size in both spider and squirrel monkeys, and the glycoform ratio in spider monkeys. In this regard it is noteworthy that, at variance with sporadic CJD VV2 or MV 2K affected subjects, both spider and squirrel monkeys are homozygous for methionine at codon 129 (Cervenáková *et al*., 1994). Therefore, our data confirm that PrP^{TSE} typing provides a molecular signature for certain prion strains within a certain host genotype, particularly when combined with genetic and neuropathological data, but is of limited value for tracing their passage among different animal species without transmission experiments.

Overall, the results of this study, combined with those obtained by other groups, indicate that at least four distinct strains of prions affect humans. According to their relative frequency in western countries they would include: (i) Strain A, related to the typical CJD phenotype or myoclonic variant (PrP^{TSE} type 1 and at least one M codon 129 allele); (ii) Strain B, related to the ataxic and kuru-plaque variants of CJD as well as kuru (PrP^{TSE} type 2 and at least one V codon 129 allele or PrP^{TSE} of 20 kDa in combination with codon 129 MM); (iii) Strain C, related to variant CJD (PrP^{TSE} type 2B and at least one M codon 129 allele); and (iv) Strain D, related to fatal insomnia, with PrP^{TSE} type 2A (sporadic form) or 2B (familial form) and at least one M codon 129 allele.

Whether additional human prion strains are related to sporadic CJD MM 2C, sporadic CJD VV1, the recently identified atypical sporadic TSE cases (Gambetti *et al*., 2008) or some rare genetic forms of as yet untransmitted prion disease remains to be determined.

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