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Neuronal and astrocytic responses involving the serotonergic system in human spongiform encephalopathies

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The relationships between the degree of cortical prion protein (PrP) deposition, tissue vacuolation and astrocytosis were studied in the frontal cortex of 27 cases of human spongiform encephalopathy, encompassing 13 cases of sporadic Creutzfeldt–Jakob disease (sCJD), four cases of familial CJD (fCJD) (one owing to E200K mutation, one owing to 144 bp insertion, one owing to P102L mutation and one owing to A117V mutation), five cases of iatrogenic CJD (iCJD) owing to growth hormone therapy and five cases of variant CJD (vCJD). The size and number of tryptophan hydroxylase (TPH) positive cells in the dorsal raphe were determined as an index of the function of the brain's serotonergic system. The amount of PrP deposited in frontal cortex in vCJD was significantly greater than that in both sCJD and iCJD, which did not differ significantly from each other. The extent of grey matter deposition of PrP correlated with that of white matter deposition. Deposition of PrP as plaques was greater in cases of sCJD bearing valine at codon 129 of PrP gene, especially when homozygous. However, all cases of vCJD displayed florid plaque formation yet these were homozygous for methionine at codon 129. Prion protein deposition as plaques was greater in cases of sCJD with 2A PrP isotype than those with 1 PrP isotype, similar to that seen in cases of vCJD all of which are 2B PrP isotype. There were no significant differences in the extent of astrocytosis between the different aetiological groups, in either grey or white matter, as visualized with glial fibrillary acidic protein (GFAP) or 5HT-2_A receptor (5HT-2_AR) immunostaining, although there was a strong correlation between the severity of 5HT-2_AR and GFAP reactions within both grey and white matter. The extent of PrP deposition within the grey, but not white, matter correlated with the degree of astrocytosis for both GFAP and 5HT-2_AR and the extent of tissue vacuolation in grey and white matter, although the latter did not correlate with degree of astrocytosis for either GFAP or 5HT-2_AR. Astrocytes may be responding directly to the presence of PrP within the tissue, rather than the vacuolar damage to neurones. Although S100β immunoreactivity was present in astrocytes in control cases, no S100β staining was seen in astrocytes in either grey or white matter in most CJD cases. There were no differences in the number of TPH-positive cells between CJD and control cases, although the mean TPH-positive cell size was significantly greater, and cells were more intensely stained, in CJD compared to controls, suggesting a pathological overactivity of the brain's serotonergic system in CJD. This may result in excessive release of 5HT within the brain triggering increased 5HT-2_AR expression within activated astrocytes leading to release and depletion of S100β protein from such cells. The clinical symptoms of fluctuating attention and arousal could be mediated, at least in part, by such alterations in function of the serotonergic system.

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Introduction

The human spongiform encephalopathies (HSE) comprise a group of clinically and pathologically heterogeneous disorders that have in common, the deposition in the brain of an abnormal fibrillar protein termed prion protein (PrP) [28]. The major clinical condition within HSE is Creutzfeldt–Jakob disease (CJD), although the group also contains inherited forms such as Gerstmann–Straussler syndrome and fatal familial insomnia, as well as transmissible disease such as Kuru [34]. Creutzfeldt–Jakob disease itself is not of single cause, and does not present with uniform clinical and pathological phenotype. There are sporadic (sCJD) and familial (fCJD) forms of the disease, as well as iatrogenically transmitted cases (iCJD) involving dural graft and growth hormone or gonadotrophin therapy [28,34]. Lastly, and most recently, a form of disease associated with transmission from cattle, known as variant CJD (vCJD) [14,37,38,42], has attained great importance.

Clinically, cases of CJD can display a wide range of signs and symptoms encompassing dementia, gait and locomotor disturbances, and (frequently transient) fluctuations in mood and level of consciousness and attention [31,36]. The underlying pathological changes are thought to stem from the (toxic effects of) deposition of PrP in the brain [28,34]. These involve a swelling and loss of neurones, producing a widespread vacuolation of the neuropil throughout cerebral cortical and subcortical regions known as 'status spongiosis', accompanied by a reactive astrocytosis. However, the pattern and distribution of the pathology between cases with different aetiologies is highly variable and their relationship to the clinical symptomatology is poorly understood.

In this study, we have therefore examined the patterns of PrP deposition and glial cell responses to this, in terms of changes in glial fibrillary acidic protein (GFAP), 5HT-2_A receptor (5HT-2_AR) and S100β proteins in 27 cases of CJD associated with differing aetiologies. We have also investigated the effects of PrP genotype and isotype on these pathological features. Lastly, we have looked for changes in the brain's serotonergic system, based upon ascending pathways from the nucleus dorsal raphe and nucleus centralis in the brainstem, because this is thought to play a pivotal role in the regulation of mood, attention and behaviour [22]. It is therefore possible that changes in this system may underpin certain of the symptoms of CJD, particular in respect of the swings in mood and consciousness common among such patients [31].

Materials and methods

Formalin-fixed blocks of frontal cortex (Brodmann areas 8/9) and brainstem at the level of the dorsal raphe nucleus (where available) were obtained from 27 cases of CJD (Table 1). Eight cases were drawn from tissue archives at University of Manchester while the remainder were acquired from the National CJD Surveillance Unit at Edinburgh. The 27 cases comprised of 13 cases of sCJD, four cases of fCJD (one owing to E200K mutation, one owing to 144 bp insertion [8], one owing to P102L mutation and one owing to A117V mutation [19]), five cases of iCJD owing to growth hormone therapy and five cases of vCJD [14]. In accordance with Health and Safety Guidelines[1] , all brains had been fixed in 10% (v/v) formalin for a minimum of 3 weeks. Dissected tissue blocks were decontaminated by immersion in 96% formic acid for 1 h and processed routinely into paraffin wax. Six neurologically and psychiatrically normal individuals of similar age, drawn from the Manchester archive, served as controls. Sections were cut at thickness of 5 μm and mounted on 5% APES-coated glass slides.

One set of sections from frontal cortex was routinely stained with haematoxylin-eosin whereas a further set was immunostained for PrP, employing a protocol recommended by CJD Unit, using the polyclonal antibody KG9 (C Birkett, Compton, UK). Briefly, sections were deparaffinized and hydrated then placed in alcoholic saturated picric acid for 15 min. Endogenous peroxidase activity was blocked by immersion in methanol containing 0.3% (v/v) hydrogen peroxide for 30 min. Sections were then successively microwaved in distilled water on high power (650 kW) for 3 × 5 min (with 5 min standing time

[1]Transmissible Spongiform Encephalopathy Agents: Safe Working and Prevention of Infection. Advisory Committee on Dangerous Pathogens. Spongiform Encephalopathy Advisory Committee.

Table 1. Selected clinical, biological and genetic data on 27 cases of Creutzfeldt–Jakob disease

Patient	*Source*	*Aetiology*	*Gender*	*Age at onset (years)*	*Age at death (years)*	*Duration (months)*	*Genetic data*	*PrP isotype*	*Clinical details*
1	Ed	Sporadic	Female	56	57	1	MM nil	1	Difficulty co-ordinating arms and legs, unsteady, unable to use left hand (numbness), chorieform movements
2	Ed	Sporadic	Female	64	65	2	MM nil	1	Disorientation, unsteadiness, faltering speech, forgetful, complaining something wrong with ear
3	Ed	Sporadic	Male	65	65	3	MV nil	2A	Progressive hearing loss (cortical deafness), unsteadiness and clumsiness affecting left arm and leg, impairment of understanding
4	Ed	Sporadic	Female	75	75	4	VV nil	2A	Unsteadiness, inappropriate behaviour and forgetfulness
5	Ed	Sporadic	Male	62	64	24	VV nil	2A	Forgetful, unsteady, pain in legs, difficulty walking
6	Ed	Sporadic	Female	63	63	9	MV nil	2A	Rapidly progressive memory loss, confusion and unsteadiness of gait, myoclonus and mixed dysphasia
7	Ed	Sporadic	Male	55	56	14	MM nil	1	Difficulty with naming and sequential activity, visual difficulty, rigidity, vacant and perplexed
8	Mc	Sporadic	Female	59	60	9	MV nil	2A	Increased tone, postural tremor, limb inco-ordination, ataxia and unsteadiness with falls, dysarthric, myoclonus.
9	Mc	Sporadic	Male	51	51	4	MM nil	1	Disturbed vision, occipital blindness, ataxic gait, rigidity, myoclonus, anxious and confused
10	Mc	Sporadic	Female	57	58	7	MM nil	1	No details available
11	Mc	Sporadic	Female	66	66	6	MM nil	2A	Memory loss, aphasic, apraxic
12	Mc	Sporadic	Female	60	61	12	VV nil	2A	Frontal lobe syndrome, inattention, perserveration
13	Mc	Sporadic	Male	50	54	53	MV nil	2A	Psychomotor slowing, perseveration, perceptuo-spatial difficulty, myoclonus
14	Ed	Variant	Female	39	41	18	MM nil	2B	Pain in legs, anxiety and irritability, feet burning, peculiar gait, memory problems, fatigue
15	Ed	Variant	Male	30	31	9	MM nil	2B	Forgetful, very emotional, aggressive, unsteady, slurred speech, hallucinations, complained of cold feet
16	Ed	Variant	Female	34	35	14	MM nil	2B	Persisting personality problems, exhaustion, not coping, forgetful, hallucinations/paranoia, unsteady, paraesthesia in feet
17	Ed	Variant	Male	18	19	12	MM nil	2B	Quieter, depressed, pain around knees, clumsy, dysarthric, ataxia, progressive dementia
18	Ed	Variant	Male	35	36	12	MM nil	2B	Odd behaviour, sleeping on floor as buttocks uncomfortable, complaining of insomnia, aggressive and anxious, complained of feeling cold, confused
19	Ed	Iatrogenic	Male	31	32	13	MV A117A	na	Progressive memory loss, ataxic gait and behavioural changes
20	Ed	Iatrogenic	Female	na	31	na	MV nil	2A	No details available

Table 1. *Continued*

Patient	*Source*	*Aetiology*	*Gender*	*Age at onset (years)*	*Age at death (years)*	*Duration (months)*	*Genetic data*	*PrP isotype*	*Clinical details*
21	Ed	Iatrogenic	Female	31	32	6	VV nil	2A	Moderate ataxia of all limbs, prominent gait ataxia
22	Ed	Iatrogenic	Male	28	29	6	VV nil	na	Asymmetrical cerebellar syndrome, myoclonus, cognitive decline, visual hallucinations, probable higher order visual dysfunction
23	Ed	Iatrogenic	Male	32	33	14	MV nil	2A	Gait disturbance, mild intellectual impairment, hand dysfunction
24	Ed	Familial	Male	57	57	4	MM E200K	na	Weight loss and personality change, dementia, dysphasia, ataxia and incontinence
25	Ed	Familial	Male	46	46	3	MV 144 bp	na	Occipital blindness, rapidly progressive cerebellar ataxia, mild personality change, thereafter confusion.
26	Ed	Familial	Female	42	45	39	VV A117V	na	Rapid intellectual decline, irritable, agitated and forgetful
27	Mc	Familial	Female	56	56	8	MV P102L	1	Writing and spelling difficulty, dysphasia, visuo- spatial problems, anxious, increased tone and clumsiness, myoclonus

PrP, prion protein; M, methionine; V, valine (MM, MV, VV represent genotypes at codon 129 of PrP gene); na, data unavailable; Ed, Edinburgh case; Mc, Manchester case.
Nil indicates no PrP gene mutation present; other polymorphic (A117A) or mutations present are indicated.

between), placed in 96% formic acid for 5 min then refrigerated in 4 M guanidine thiocyanate for 2 h. Following washing in distilled water, sections were incubated with normal rabbit serum (1 : 5) for 20 min then incubated overnight in primary antibody at dilution of 1 : 150 at 4°C. Secondary (biotinylated rabbit antimouse) antibody (1 : 200) was applied for 30 min at room temperature, then immunostained with avidin-biotin kit (Vector), visualized with DAB and lightly counterstained with haematoxylin. Other sections from frontal cortex were immunostained for 5HT-2_AR protein as described elsewhere [40] using a well-characterized monoclonal antibody [39] at a dilution of 1 : 100, following brief microwaving [2 × 5 min at full power (650 kW)] in 0.1 M citrate buffer pH 7.35. Finally, further sets of sections from frontal cortex were immunostained for GFAP (Dako) (again after microwaving as above) at a dilution of 1 : 750, or for S100β (Sigma) at a dilution of 1 : 500. All incubations in primary antibody were performed overnight at 4°C and sites of immunoreaction visualized using a standard ABC procedure with DAB as chromogen.

Sections of brainstem were immunostained for tryptophan hydroxylase (TPH), as a marker of serotonergic neurones, using the primary antibody PH8 (Pharmingen) at dilution of 1 : 500, following microwaving in 0.1 M citrate buffer pH 6.0 (as above).

The codon 129 polymorphism in PrP gene was determined from DNA extracted from frozen brain tissue, using the method described by Owen *et al.* [23]. Prion protein isotype was determined by Western blotting according to a variation [14] on the method of Collinge *et al.* [9]. In this, PrP isotypes are classified [25] according to mobility variations corresponding to different points of proteinase K-mediated N-terminal truncation and the relative extent of glycosylation at two sites within the PrP molecule. Three patterns emerge. Type 1 relates to a PrP fragment of approximately 21 kDa with a predominance of monoglycosylated isoforms. Type 2 relates to a smaller fragment of approximately 19 kDa and this can be further categorized into type 2A with predominance of PrP isoforms with one glycosylated site occupied and type 2B with predominance of isoforms with both sites glycosylated [14].

The severity of pathological changes within frontal cortex (i.e. PrP deposition, changes in GFAP and 5HT-2_AR, tissue vacuolation) was rated semiquantitatively on a 6-point scale: 0 = no change; 1 = very mild, isolated changes;

2 = mild, uniform changes; 3 = moderate change; 4 = severe change; 5 = very severe change. Grey and white matter was assessed separately. The extent of each pathological change was rated by two observers (E.F. and D.M.A.M.) and the degree of concordance was performed using a nonparametric correlation (Spearman rank correlation) method. In all instances, a highly significant ($P < 0.001$) correlation was achieved between the two sets of observations. Relationships between PrP deposition, GFAP and 5HT-2_AR expression and tissue vacuolation were also examined by Spearman rank correlation. Differences in PrP deposition, GFAP and 5HT-2_AR staining and tissue vacuolation between the various aetiological subgroups, and between genotype and isotype groups were compared by nonparametric ANOVA (Kruskal–Wallis) with *post hoc* Mann–Whitney *U*-test to determine significance of differences in mean values, when ANOVA result was $P < 0.05$ significance level.

The size and number of TPH-positive cells in sections of brainstem from 14 cases of CJD and the six control cases were determined using Leica Quantimet 550s Image Analysis system (Cambridge, UK). The TPH-immunostained sections were viewed under a Leica DMRB microscope at × 10 magnification and the images captured electronically using Sony 93P camera. The image was analysed using in house software with the TPH-positive cells being optimally separated from the background by thresholding for colour and density level, and size, such that only those objects within the measuring field greater than 130 μm^2 were selected for counting and size measurement. The size of the measuring field was determined manually so as to enclose only that area of brainstem containing TPH-positive structures. To ensure comparability of sampling, only sections also containing the pigmented cells in the rostral part of the noradrenergic locus caeruleus were used, hence limiting measurement to 14/27 CJD cases. Differences in cell size and number between each of the aetiological groups and control cases were compared by Mann–Whitney *U*-test.

Results

Prion protein gene codon 129 polymorphism and prion protein isotypes

No mutations in the PrP gene were found in the 13 sCJD cases; six cases were MM (M = methionine) genotype at codon 129, four were MV (V = valine) genotype and three were VV genotype (Table 1). All cases of vCJD bore MM genotype. Three cases of iCJD bore MV genotype (one case no. 20 with silent A117A polymorphism) and two were VV genotype. The fCJD case with E200K mutation (case no. 24) was MM genotype, the 144 bp insertion case (case no. 25) was MV genotype, the A117V case (case no. 26) was VV genotype and the P102L case (case no. 27) was MV genotype.

Prion protein isotyping was available for all 13 sCJD, all five vCJD, 3/5 iCJD cases and 1/4 fCJD cases. Consistent with previous large surveys [14,24,26], 5/6 present cases of sCJD with codon 129 MM genotype showed PrP 1 isotype and 1/6 with PrP 2A isotype, with the remaining seven sCJD cases (MV or VV genotypes) all being PrP 2A isotype. Those three cases of iCJD with MV or VV genotypes all showed PrP 2A isotype. The five cases of vCJD with MM genotype were all of PrP 2B isotype, and the P102L fCJD case was PrP 1 isotype (Table 1).

Prion protein distribution and amount

There was much variation in both the extent and the distribution of the PrP deposition in the frontal cortex of the 27 cases of CJD. Deposition was mostly within grey matter and except for the one case with A117V mutation was only rarely present in white matter. Two cases of sCJD showed no deposition of PrP in any part of the frontal cortex, while in two other sCJD cases, the fCJD case with 144 bp insertion and in 1/5 iCJD cases deposition was rare.

Several patterns of deposition were observed. Most common was a perivacuolar deposition that was often concentrated (coalesced) into small and larger plaque-like structures that blended with the finer perivacuolar deposits (Figure 1**a**). This pattern was present in all of the 11 positive sCJD cases. Heavy PrP deposition was characteristically seen in all five vCJD cases. This typically occurred as numerous small cluster plaques (Figure 1**c**) along with amorphous pericellular and perivacuolar deposition. In the iCJD cases there were only scant plaque-like deposits spread throughout the cortex (Figure 1**d**). In the fCJD case, associated with E200K mutation, PrP was spread as a uniformly fine deposit throughout all cortical layers. In the P102L fCJD case, a more discrete and heavy plaque formation was observed (Figure 1**b**), chiefly in the deeper cortical layers, along with the finer perivacuolar deposits in these and other cortical layers. In the fCJD case associated with A117V mutation, there were large plaques and perivascular deposits mostly within the deeper cortical layers and arranged in a linear fashion along the cortical ribbon, with

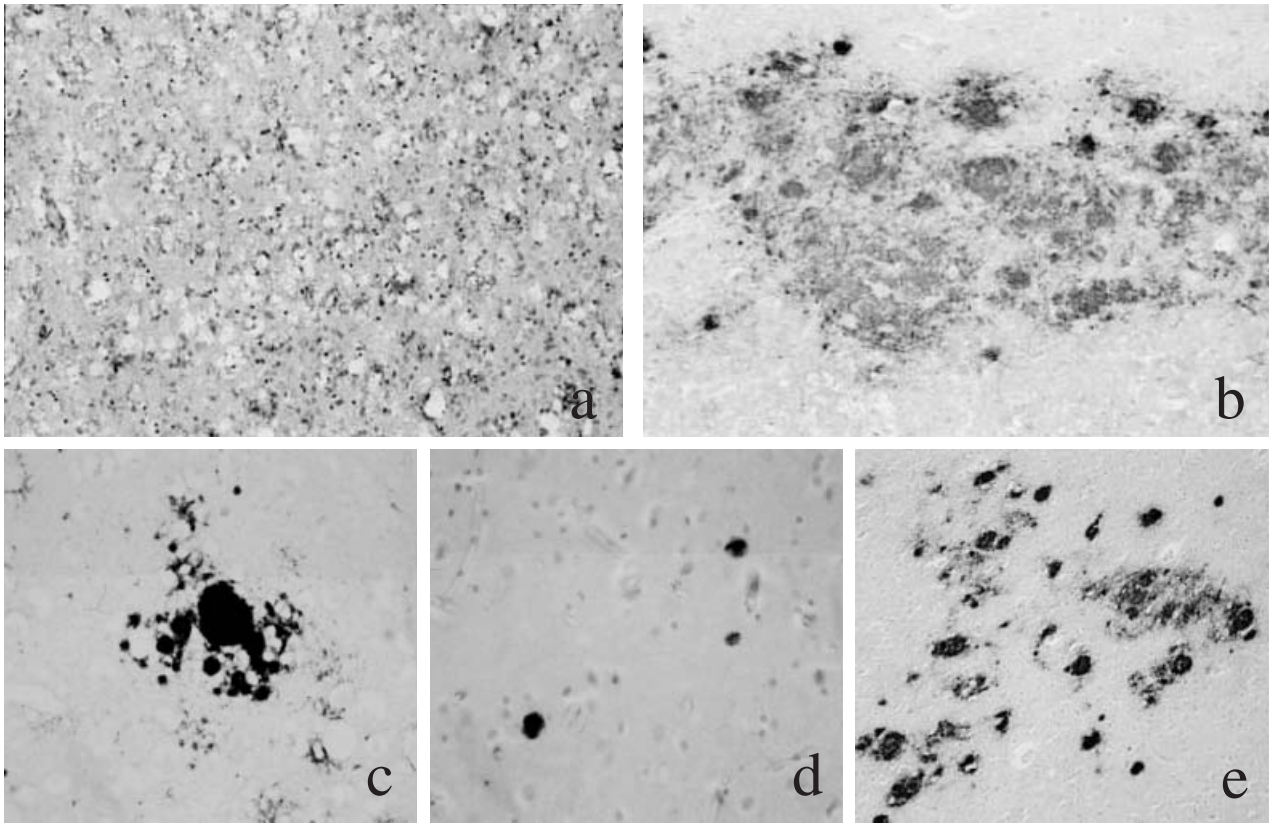

Figure 1. Prion protein (PrP) deposition in the frontal cortex in different aetiological forms of Creutzfeldt–Jakob disease (CJD). In sporadic CJD (**a**), there is perivacuolar deposition of PrP, in which fine deposits blend with small and larger plaque-like structures. In variant CJD (**c**), PrP deposition characteristically occurs as numerous small cluster plaques along with amorphous pericellular and perivacuolar deposits (**b**). In iatrogenic CJD (**d**), scant plaque-like deposits are spread throughout the cortex. In familial CJD associated with P102L mutation (**b**), heavy plaque formation is observed mostly in deeper cortical layers, along with the finer perivacuolar deposits, whereas in A117V mutation, there are large plaques and perivascular deposits, again mostly within the deeper cortical layers and arranged in a linear fashion along the cortical ribbon, with smaller plaques and much fine deposit elsewhere through the cortex (**e**). Magnification: (**a**,**b**,**e**) × 10, (**c**,**d**) × 25.

smaller plaques and much fine deposit elsewhere through the cortex (Figure 1**e**). Plaques were also quite common in the white matter of this case.

The amount of PrP deposited within the frontal cortex in cases of vCJD was significantly greater than that in both sCJD ($P < 0.05$) and iCJD ($P < 0.01$) (fCJD not analysed), but there were no significant differences in PrP deposition between the other aetiological groups (Figure 2**a**). The extent of grey matter deposition of PrP across all aetiological cases correlated with that of white matter deposition ($r_s = 0.467$, $P < 0.01$).

Relationship to prion protein genotype

The presence and severity of PrP deposition within the frontal cortex was investigated with respect to the codon 129 M/V polymorphism in the prion gene. Over all 27 cases, there were no significant differences in the degree of PrP deposition between MM, MV and VV genotypes or between bearers and nonbearers of valine at codon 129 (Table 2). However, pooling cases in this way masked important differences between and within aetiological groups (Table 2). The degree of PrP deposition and extent of plaque formation (especially) tended to be greater ($P = 0.07$) in cases of sCJD bearing MV and VV genotypes at codon 129, than in those with MM genotype; homozygosity for valine did not appear to increase the extent of PrP deposition compared to valine heterozygotes (Table 2). Despite bearing MM genotype, all vCJD cases showed extensive PrP deposition and florid plaque formation (Figure 1**c**, Table 2). All five iCJD cases bore at least one V allele, with mild to moderate PrP deposition in frontal cor-

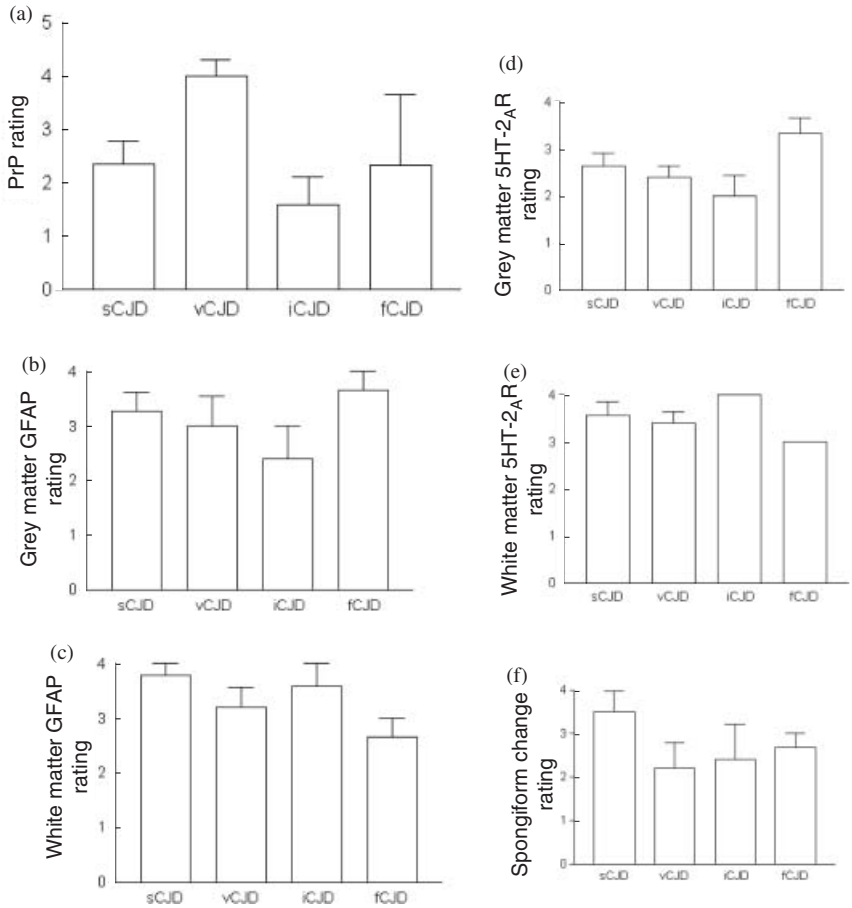

Figure 2. Mean ± SD rating of prion protein (PrP) deposition (**a**), glial fibrillary acidic protein (GFAP) (**b**,**c**) and 5HT-2_AR immunoreaction (**d**,**e**) activity in grey (**b**,**d**) and white (**c**,**e**) matter, and spongiform change (**f**) for cases of sporadic Creutzfeldt–Jakob disease (sCJD), variant CJD (vCJD), iatrogenic (iCJD) and familial CJD (fCJD).

tex of the four MV cases (Figure 1**c**), but only rare PrP deposition in the single VV case. Of the four fCJD cases, mild PrP deposition was present in the MM E200K mutation (Figure 1**e**) and in the MV 144 bp insertion, cases, but there was extensive plaque formation in the P102L and A117V mutation cases that were valine heterozygous and homozygous, respectively (Figures 1**b**,**e**, Table 2).

Relationship to prion protein isotype

Across all aetiological types, cases with PrP 2A and 2B isotypes (separately and combined) showed a greater ($P < 0.05$) PrP deposition, in the form of plaques, than cases with PrP 1 isotype (Table 2). The PrP 2B isotype was exclusive to vCJD, and these cases showed a greater ($P = 0.01$) deposition of PrP than cases of sCJD and iCJD (separately and combined) with PrP 2A isotype. Cases of sCJD alone with 2A isotype, however, did not display greater ($P < 0.05$) PrP deposition than those with PrP 1 isotype (Table 2).

Astrocytosis

Reactive astrocytosis, as evidenced by GFAP immunoreaction, was also highly variable within the grey matter of the frontal cortex. Most commonly, there was a mild to moderate subpial reaction with a variable (mild to severe) perivacuolar distribution (Figure 3**a**). On other occasions, however, astrocytosis was mild, patchy and occurred in clusters around normal appearing large and small blood vessels (Figure 3**c**). When the PrP was present as large plaques, astrocytosis was florid and frequently clustered in and around the PrP deposits (Figure 3**e**). All cases showed astrocytosis within the white matter, this ranging from

Table 2. Mean ± SD rating value for deposition of prion protein (PrP), extent of astrocytosis and spongiosis in 27 cases of Creutzfeldt–Jakob disease (CJD) according to codon 129 genotype and PrP isotype

		Genotype				*Isotype*		
		MM	*MV*	*VV*	*MV + VV*	*1*	*2A*	*2B*
PrP	sCJD	1.3 ± 1.5 (6)	3.0 ± 1.6 (4)	2.6 ± 0.6 (3)	2.8 ± 1.2 (7)	1.4 ± 1.7 (5)	2.8 ± 1.1 (8)	
	vCJD	4.0 ± 0.7 (5)						4.0 ± 0.7 (5)
	iCJD		2.3 ± 0.6 (3)	0.5 ± 0.7 (2)	1.6 ± 1.1 (5)		1.6 ± 0.6 (3)	
	fCJD	1.0 (1)	3.0 ± 2.8 (2)	5.0 (1)	3.6 ± 2.3 (3)	5.0 (1)		
	All cases	2.4 ± 1.8 (12)	2.7 ± 1.5 (9)	2.3 ± 1.8 (6)	2.6 ± 1.6 (15)	2.0 ± 2.1 (6)	2.4 ± 1.1 (11)	4.0 ± 0.7 (5)
GFAP	sCJD	2.6 ± 1.5 (6)	3.8 ± 1.3 (4)	3.7 ± 0.6 (3)	3.7 ± 1.0 (7)	2.6 ± 1.6 (5)	3.2 ± 0.7 (8)	
	vCJD	3.0 ± 1.2 (5)						3.0 ± 1.2 (5)
	iCJD		2.3 ± 0.6 (3)	1.0 ± 0 (2)	1.8 ± 0.8 (5)		2.3 ± 1.2 (3)	
	fCJD	4.0 (1)	3.0 ± 1.4 (2)	3.0 (1)	3.0 ± 1.0 (3)	5.0 (1)		
	All cases	2.9 ± 1.3 (12)	3.1 ± 1.2 (9)	2.7 ± 1.4 (6)	2.9 ± 1.2 (15)	3.0 ± 1.8 (6)	3.0 ± 1.0 (11)	3.0 ± 1.2 (5)
5HT-2_AR	sCJD	2.3 ± 0.5 (6)	2.0 ± 0.8 (4)	3.3 ± 0.6 (3)	2.6 ± 1.0 (7)	2.2 ± 0.4 (5)	2.8 ± 1.0 (8)	
	vCJD	2.4 ± 0.6 (5)						2.4 ± 0.6 (5)
	iCJD		2.6 ± 0.6 (3)	1.5 ± 0.7 (2)	2.2 ± 0.8 (5)		2.0 ± 1.0 (3)	
	fCJD	3.0 (1)	4.5 ± 0.7 (2)	3.0 (1)	4.0 ± 1.0 (3)	5.0 (1)		
	All cases	2.4 ± 0.5 (12)	2.8 ± 1.2 (9)	2.7 ± 1.0 (6)	2.8 ± 1.1 (15)	2.6 ± 1.2 (6)	2.6 ± 1.2 (11)	2.4 ± 0.6 (5)
SPONG	sCJD	3.2 ± 2.3 (6)	4.0 ± 1.4 (4)	4.3 ± 0.6 (3)	4.1 ± 1.1 (7)	2.8 ± 2.3 (5)	4.3 ± 1.0 (8)	
	vCJD	2.4 ± 1.1 (5)						2.4 ± 1.1 (5)
	iCJD		2.3 ± 0.6 (3)	0.5 ± 0.7 (2)	1.6 ± 1.1 (5)		2.7 ± 1.5 (3)	
	fCJD	3.0 (1)	4.0 ± 1.4 (2)	1.0 (1)	3.0 ± 2.0 (3)	1.0 (1)		
	All cases	2.6 ± 1.6 (12)	3.4 ± 1.3 (9)	2.5 ± 2.0 (6)	3.1 ± 1.7 (15)	2.5 ± 2.2 (6)	3.8 ± 1.3 (11)	2.4 ± 1.1 (5)

sCJD, sporadic CJD; vCJD, variant CJD; iCJD, iatrogenic CJD; fCJD, familial CJD; GFAP, glial fibrillary acidic protein; 5HT-2_AR, 5HT-2_A receptor protein; SPONG, spongiosis; M, methionine; V, valine allele at codon 129 of prion protein gene.
Number of cases examined in parentheses.

mild to very severe (Figure 3**g**). 5HT-2_AR immunoreaction was exclusively located within reactive astrocytes and followed an equivalent distribution to that of GFAP within the same cases (Figures 3**b**,**d**,**f**,**h**, respectively).

There were no significant differences in the extent of astrocytosis between the different aetiological groups, in either grey or white matter, as visualized with GFAP (Figures 2**b**,**c**) or 5HT-2_AR (Figures 2**d**,**e**) immunostaining. Nonetheless, across all aetiological groups, there was strong correlation between the severity of 5HT-2_AR and GFAP reactions within both grey ($r_s = 0.631$, $P < 0.001$) and white ($r_s = 0.510$, $P < 0.001$) matter. However, the degree of grey matter astrocytosis did not correlate with that within white matter for either GFAP ($r_s = 0.262$, $P > 0.05$) or 5HT-2_AR ($r_s = 0.142$, $P > 0.05$).

The presence and severity of astrocytosis, as detected by GFAP and 5HT-2_AR immunostaining, within the frontal cortex was also investigated with respect to the codon 129 M/V polymorphism in the prion gene and PrP isotype. Overall, there were no significant differences in the degree of astrocytosis, for either GFAP or 5HT-2_AR immunostaining between MM, MV and VV genotypes or between bearers and nonbearers of valine at codon 129 (Table 2). However, in sCJD, as with PrP deposition, astrocytosis (GFAP immunoreactivity) tended to be greater ($P = 0.1$) in bearers of valine at codon 129 than nonbearers (Table 2). There were no significant differences, overall, in the degree of astrocytosis, for either GFAP or 5HT-2_AR immunostaining between bearers of PrP 1, 2A or 2B isotypes (Table 2).

Tissue vacuolation

In haematoxylin-eosin staining, vacuolation (spongiform change) was, in most instances, seen in the grey matter of the frontal cortex but only rarely and patchily in white matter. The severity of this was highly variable, ranging from a mild microvesicular change (Figure 4**a**) through to a florid and confluent cavitation (Figure 4**b**). Vacuolation was sometimes present throughout the full depth of the cortical ribbon although more commonly was restricted to specific layers, generally coinciding with sites of PrP deposition. There were no significant differences in the extent of vacuolation between the different aetiological groups (Figure 2**f**).

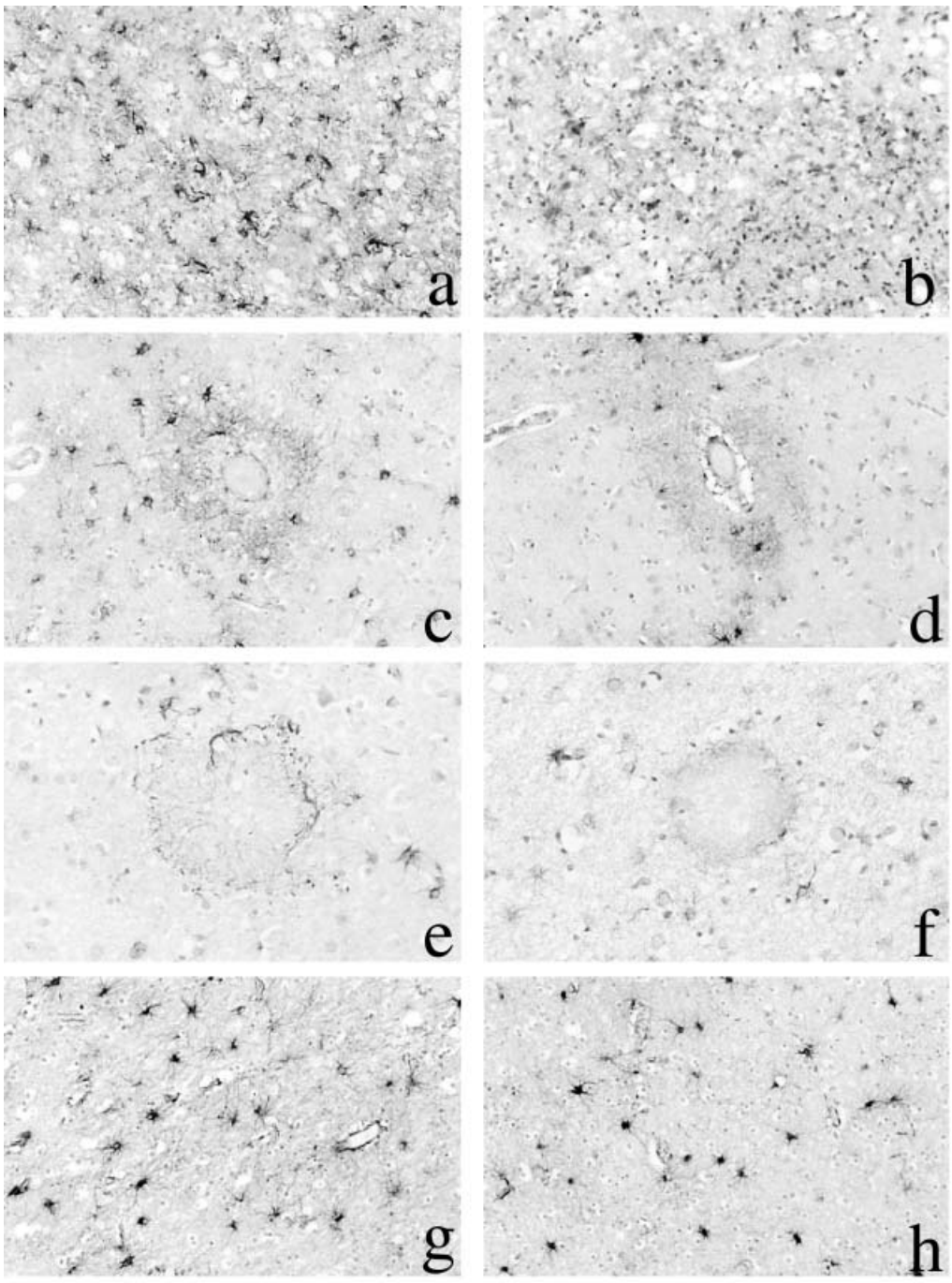

Figure 3. Reactive astrocytosis in the frontal cortex in different aetiological forms of Creutzfeldt–Jakob disease (CJD). In glial fibrillary acidic protein (GFAP) immunoreaction (**a**,**c**,**e**,**g**) reactive astrocytosis is highly variable. In grey matter of the frontal cortex in sporadic CJD, there was usually a mild to moderate subpial reaction with a variable (mild to severe) perivacuolar distribution (**a**). However, on other occasions, astrocytosis was mild, patchy and occurred in clusters around normal appearing large and small blood vessels (**c**). When the PrP was present as large plaques (as in familial CJD), astrocytosis was florid and frequently clustered in and around the PrP deposits (**e**). All cases, irrespective of aetiology, showed astrocytosis within the white matter, ranging from mild to severe (**g**). 5HT-2_A immunoreaction was exclusively located within reactive astrocytes and followed an equivalent distribution to that of GFAP within the same cases (**b**,**d**,**f**,**h**, respectively). All × 10 magnification.

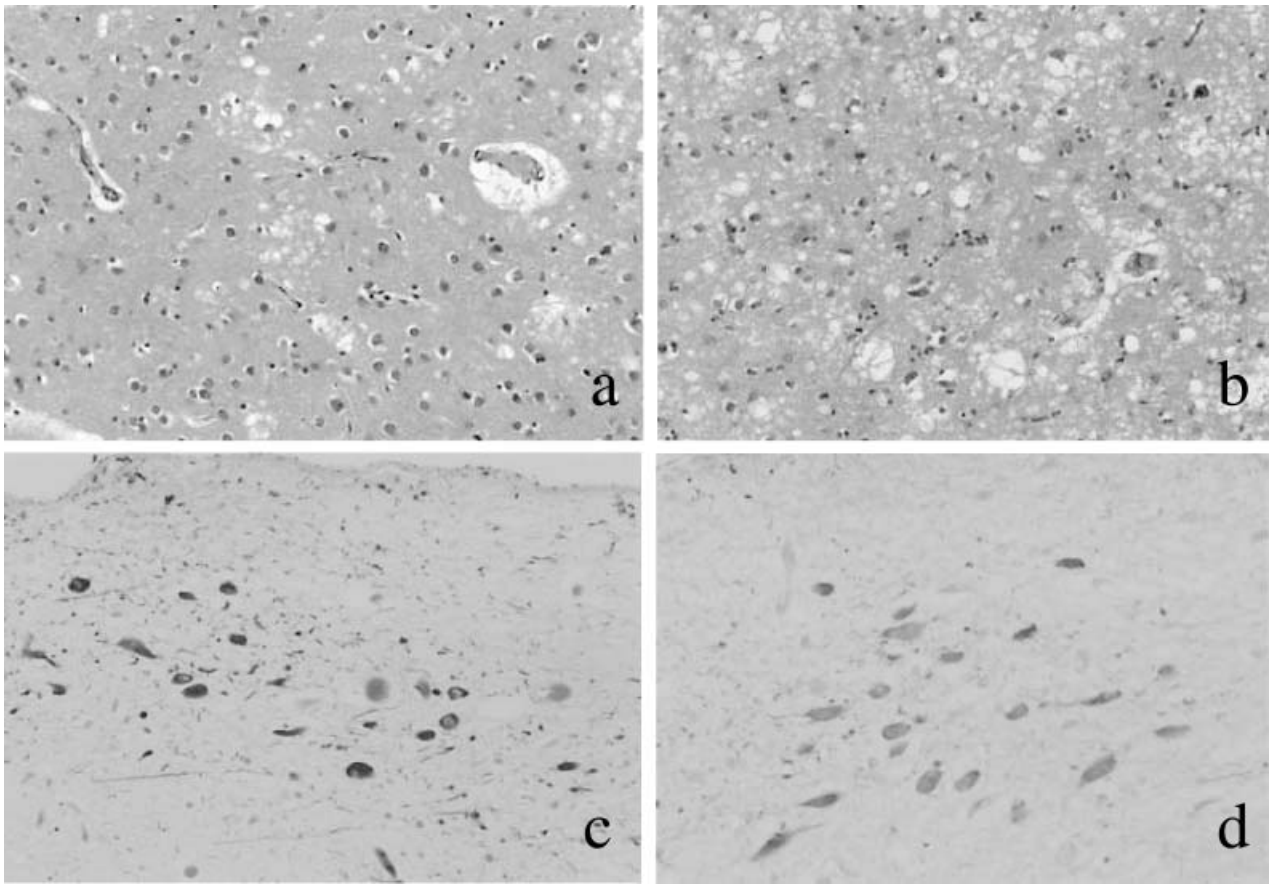

Figure 4. Tissue vacuolation (spongiform change) was seen in the grey matter of the frontal cortex in most cases, but only rarely and patchily so in white matter. The severity of spongiform change was highly variable, ranging from a mild microvesicular change (**a**) through to a florid and confluent cavitation (**b**). Tryptophan hydroxylase-positive cells in the upper brainstem in CJD cases are seen as clusters of medium and large cells with fusiform or multipolar cell bodies, usually with long, slender dendritic processes, although other times processes are short and stumpy (**c**). Cells appear to be more intensely stained in CJD (**c**), than in control (**d**) cases. All × 10 magnification.

Again, there were no overall significant differences in the degree of spongiosis between MM, MV and VV genotypes or between bearers and nonbearers of valine at codon 129 (Table 2), although in sCJD, as with astrocytosis and PrP deposition, spongiosis was no greater in bearers of valine at codon 129 than in nonbearers (Table 2). There were no significant differences, overall, in the degree of spongiosis between bearers of PrP 1, 2A or 2B isotypes (Table 2).

S100β immunoreactivity

S100β immunoreactivity was present in all six controls, staining astrocytes within subpial regions and (patchily) surrounding blood vessels of grey and white matter in a similar pattern to GFAP and 5HT-2_AR antibodies (not shown). In contrast, there was no staining for S100β in either grey or white matter in 14/17 CJD cases examined. Occasional astrocytes in the white matter, close to the junction with the grey matter, were stained weakly in 2/3 CJD cases and moderately strongly so in the other CJD case. The cell bodies of these immunopositive astrocytes were uniformly stained with little staining of cell processes (cf. GFAP and 5HT-2_AR reactions). There was no correlation between S100β and GFAP or 5HT-2_AR reactions in the CJD cases.

Relationships between prion protein, vacuolation and astrocytosis

Across all aetiological groups, the extent of PrP deposition within the grey matter correlated with the degree of astro-

cytosis for both GFAP ($r_s = 0.422$, $P = 0.028$) and 5HT-2_AR ($r_s = 0.457$, $P = 0.016$). Astrocytosis within the white matter did not correlate with PrP for either GFAP ($r_s = 0.061$, $P > 0.05$) or 5HT-2_AR ($r_s = 0.260$, $P > 0.05$). The degree of PrP deposition also correlated with the extent of tissue vacuolation for both grey ($r_s = 0.645$, $P < 0.001$) and white ($r_s = 0.496$, $P < 0.001$) matter. The extent of tissue vacuolation in the grey matter did not correlate with degree of astrocytosis for either GFAP or 5HT-2_AR, in neither grey nor white matter.

Tryptophan hydroxylase immunohistochemistry

Tryptophan hydroxylase-positive cells in the upper brainstem in CJD cases were easily identified as clusters of medium and large cells with fusiform or multipolar cell bodies located either side of the midline (Figure 4**c**). Long, slender dendritic processes emanated from the perikaryon in some instances, although in others processes were short and stumpy. Cells appeared to be more intensely stained in the CJD cases (Figure 4**c**) than in those from normal controls (Figure 4**d**). Cell counting showed no overall significant differences ($P = 0.2$) in TPH-positive cell number between CJD (53.7 ± 42.3) and control (54.0 ± 45.1) cases, although the number of TPH-positive cells in vCJD (25.2 ± 16.4) was less ($P = 0.05$) than that in the other aetiological variants (65.1 ± 44.7). The mean TPH-positive cell size tended to be greater ($P = 0.1$) in CJD ($257.2 \pm 59.0\,\mu m^2$) compared to controls ($208.6 \pm 40.7\,\mu m^2$), although there were no significant variations in TPH-positive cell size between the CJD aetiological groups.

Discussion

The pathological mechanisms responsible for the deposition of insoluble PrP, reactive astrocytosis and vacuolation (spongiform change) in CJD, and the factors that determine their form and distribution, are still poorly understood.

Previous studies in sCJD [12,24,26,27,32] have suggested that deposition of PrP, often as well-formed (sometimes kuru-type) plaques, is enhanced in patients bearing MV or VV genotypes for codon 129 polymorphism of PrP gene. Present data from the 13 sCJD cases studied here in which a greater deposition of PrP was present in bearers of at least one valine allele at codon 129 are consistent with this suggestion. However, this 'rule' may not universally apply across all aetiological forms of CJD. For example, all five vCJD cases showed the florid plaque formation characteristic of this particular aetiological form of CJD yet, as with all other cases of vCJD so far reported [14,38], these were of codon 129 MM genotype. Moreover, all five iCJD cases examined here bore at least one valine allele at codon 129 yet both of the VV cases showed hardly any PrP deposition within frontal cortex. Nonetheless, as in the three other iCJD cases with MV genotype, these codon 129 VV cases showed many plaques within the cerebellum, the brain region principally affected in this aetiological form of CJD [6]. Because nearly all UK iCJD cases associated with growth hormone treatment (including those cases studied here) have codon 129 MV or VV genotypes [6], as in vCJD, it is not possible for us to judge, presently, whether it is the possession of valine at codon 129, *per se*, that influences the pattern of PrP plaque formation in this aetiological form of CJD or whether there are other influences such as PrP isotype. Of the four fCJD cases studied here, the case with A117V mutation showed severe plaque formation and bore codon 129 VV genotype. However, a florid plaque formation is typical of this particular mutation [18–20], and might still occur irrespective of codon 129 genotype. Interestingly, however, no cases homozygous for methionine at codon 129 in this particular mutation have as yet been described, either in the kindred from which the present case was drawn [19] or from other kindreds bearing the same mutation [18,20], although in one kindred [19], an unaffected individual who had elected for genetic screening was homozygous for methionine, suggesting that the A117V mutation may be in complete linkage dysequilibrium with valine at codon 129 of the affected gene with both allelic variations being inherited from an ancestral founder as part of an extended haplotype in and around PrP gene [19]. The P102L case typically showed extensive, florid plaque formation and was MV genotype while two other fCJD cases bore sparse PrP, even though the case with 144 bp insertion had codon 129 MV genotype. Hence, in the present study, as in previous more extensive surveys on sCJD [12,14,26,27,32], PrP deposition as plaques in fCJD is broadly favoured by possession of valine at codon 129, especially valine homozygosity.

However, as we have seen, possession of valine at codon 129 is not mandatory for plaque formation in CJD as this can occur, as in vCJD, even when valine is not present. Although the present sample is limited in size, we detected clear differences in the extent and morphological characteristics of PrP deposition, especially as plaques, in bearers

of PrP isotypes 1 and 2, with greater plaque formation in type 2 cases, irrespective of whether they were type 2A as in sCJD and iCJD or type 2B as in vCJD. These data indicate that PrP glycosylation state may also be important in plaque formation in CJD with mono- and diglycosylated forms of PrP being able to assemble into plaques more avidly than nonglycosylated forms. However, because there are (relatively few) MM genotype cases with isotype 2 and MV and VV cases with isotype 1 that do not form PrP plaques [23], pathological differences in form and distribution of PrP deposition may, as suggested by Parchi *et al*. [24,26], be determined by a combination of genotype at codon 129 and the relative glycosylation state of PrP.

The results of this present study show that there are strong correlations between deposition of PrP and extent of astrocytosis on one hand, and the degree of tissue vacuolation on the other, implying that these pathological changes may be causally related. Consistent with present findings, Armstrong *et al*. [1] have reported that the spatial pattern of vacuolation correlates in sCJD with the pattern of deposition of PrP, although such a relationship was not seen in cases of vCJD [14]. Spongiform change relates to a swelling of neuronal perikarya, processes and synapses, with loss of organelles and membranes. It is possible therefore that the pattern of vacuolation [1], like that of PrP deposition [2], may follow well-defined pathways and lie within distinct cortical columns. The correlation between PrP deposition and GFAP/5HT-2_AR immunoreactivity is indicative of reactive changes on the part of astrocytes to tissue injury. The lack of correlation between tissue vacuolation suggests that the astrocytes are reacting directly to the deposition of PrP and not indirectly through the effects of this upon nerve cell structure and function. The observations both here, and experimentally [41], of astrocytes close to prion plaques, encircling such deposits with their processes, is consistent with such a viewpoint. Indeed, a synthetic neurotoxic PrP peptide induces *in vitro* astroglial proliferation by a mechanism independent of its effects on neurones [10]. Astrocytes perform a complex portfolio of functions, enlarging their cell body and increasing the number, size and branching complexity of their processes upon activation. Increased GFAP may reflect their role as principal cells responsible for repair and scar formation within the brain, isolating PrP plaques and more diffuse deposits from the healthy tissue.

Although a role in tissue repair might explain, at least in part, the activation of astrocytes when there is deposition of insoluble proteins within the brain, be it PrP in CJD or amyloid β protein in Alzheimer's disease and Down's syndrome, such a role is unlikely to explain the elevation of 5HT-2_AR expression in astrocytes in other reactive situations, such as stroke [40]. Experimental studies [41] have suggested that astrocytes may produce or accumulate PrP, even before neuronal degeneration sets in, this then leading to further astrocytosis and continuing PrP formation and deposition. Astrocytosis may therefore play an active part in the disease pathogenesis, contributing to PrP deposition and neurodegeneration, and not simply being reactive to the presence of PrP. The presence of receptors for classical neurotransmitters upon astrocytes has long been recognized [16], and in pathological states the transmitter uptake systems of neurones may become impaired and the need for homeostasis, with respect to neurotransmitters like 5HT, be increased [21]. However, 5HT re-uptake in neurones is mediated by 5HT-1 receptors and not 5HT-2 receptors [44], implying an alternative reason for this increased expression. When stimulated by 5HT via 5HT-2_AR, astrocytes mobilize glycogen stores and produce more glucose [7]. This may be necessary to provide more energy for the activated astrocyte itself or it may be in part required to support the metabolic needs of injured (in this case by PrP deposition) neurones. Hence, it is possible that changes in 5HT-2_A in astrocytes are indicative of an excess of 5HT within the extracellular fluid. The present finding of an increase in the size and staining of TPH-positive cells in the dorsal raphe (the source of the brain's 5HT system) similarly suggests a pathologically raised 5HT production. Interestingly, protein 14-3-3, a regulatory molecule for several proteins associated with the cell cycle and apoptosis, is increased in cerebrospinal fluid of patients with all aetiological forms of CJD [4,5,11,15,43]. 14-3-3 protein has been shown to increase TPH activity and 5HT levels [3]. Indeed, the number of TPH-positive cells is increased in patients with another form of prion disease, familial fatal insomnia [33]. Moreover, in scrapie-infected guinea pigs, there is increased 5HT-mediated activation of adenylate cyclase [30]. All of these data point towards an enhanced serotonergic transmission in CJD. It is known that 5HT is responsible for triggering the release of S100β from astrocytes into the extracellular space via stimulation of their 5HT-1_A receptors [35], whereas depletion of 5HT through inhibition of TPH activity results in the accumulation of S100β in cortical astrocytes [29]. After 5HT normalization, S100β levels also return to normal [29]. In this present study, we were unable to detect S100β protein in astrocytes in most cases of CJD yet were able to demonstrate

this in control cases. This suggests astrocytes in CJD may be depleted of S100β, further evidence of an overactive 5HT system. Indeed, consistent with these immunohistochemical findings are the observations that S100β levels in cerebrospinal fluid are increased [4,11] to an extent that this might also be of diagnostic relevance [17]. Released S100β may participate in attempts to stabilize the cytoskeleton in damaged cortical neurones [13], or stimulate regeneration or repair of synaptic terminals [35].

In summary, therefore, in this present study, we have presented evidence of an impairment of the brain's serotonergic system in CJD leading to its overactivity. What the functional consequences of this might be is not known. Through its widespread distribution of nerve terminals, the serotonergic system is placed to influence many brain functions. Cognitive and psychiatric disturbances are often observed in CJD [31,36] and may be the dominant clinical feature, especially in vCJD where delusions and hallucinations are common [38,42]. Attention and arousal are compromised, often in a strikingly fluctuating way [31]. It is possible that such symptoms are mediated, at least in part, by alterations in function (an overactivity) of the serotonergic system. Further studies are needed to fully investigate the role of the serotonergic system in CJD, and to evaluate what the functional effects of such changes might be.

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