Quantification of surviving cerebellar granule neurones and abnormal prion protein (PrPSc) deposition in sporadic Creutzfeldt–Jakob disease supports a pathogenic role for small PrPSc deposits common to the various molecular subtypes

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Aims: Neuronal death is a major neuropathological hallmark in prion diseases. The association between the accumulation of the disease-related prion protein (PrPSc) and neuronal loss varies within the wide spectrum of prion diseases and their experimental models. In this study, we investigated the relationships between neuronal loss and PrPSc deposition in the cerebellum from cases of the six subtypes of sporadic Creutzfeldt–Jakob disease (sCJD; n = 100) that can be determined according to the M129V polymorphism of the human prion protein gene (PRNP) and PrPSc molecular types. Methods: The numerical density of neurones was estimated with a computer-assisted image analysis system and the accumulation of PrPSc deposits was scored. Results: The scores of PrPSc immunoreactive deposits of the punctate type (synaptic type) were correlated with neurone counts – the higher the score the higher the neuronal loss – in all sCJD subtypes. Large 5- to 50-μm-wide deposits (focal type) were found in sCJD-MV2 and sCJD-VV2 subtypes, and occasionally in a few cases of the other studied groups. By contrast, the highest scores for 5- to 50-μm-wide deposits observed in sCJD-MV2 subtype were not associated with higher neuronal loss. In addition, these scores were inversely correlated with neuronal counts in the sCJD-VV2 subtype. Conclusions: These results support a putative pathogenic role for small PrPSc deposits common to the various sCJD subtypes. Furthermore, the observation of a lower loss of neurones associated with PrPSc type-2 large deposits is consistent with a possible ‘protective’ role of aggregated deposits in both sCJD-MV2 and sCJD-VV2 subtypes.

Keywords: cerebellum, Creutzfeldt–Jakob disease, neuronal loss, prion protein, PRNP codon 129 polymorphism, PrP deposition
Introduction

The human prion diseases are neurodegenerative diseases that are transmissible. They can be sporadic, inherited or acquired by infection and include Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome, kuru, fatal insomnia and variably protease-sensitive prionopathy [1,2]. These diseases are characterized by progressive dysfunction and neuropathological changes that include spongiform degeneration in the grey matter, neuronal loss, reactive astrogliosis, microglial proliferation, the deposition of altered forms of the prion protein (PrP) in the brain and, in a minority of cases, the presence of congophilic amyloid plaques [1,3].

Neuronal death is a central component of the neuropathology of prion diseases [4] and the accumulation of neurotoxic disease-associated isoforms of prion protein (PrPSc; insoluble and protease-resistant higher order aggregates of the physiological PrP protein) in the brain has been proposed to be an early causative event of neurodegeneration [5]. The neurotoxic properties of functional domains of the PrP molecule have been investigated in in vitro cell culture and transgenic mice models, but many questions remain unresolved. Neuronal death can be induced in the presence or absence of detectable abnormal PrP [6–9]. Despite intensive research efforts, the mechanisms of neurodegeneration are not well understood, especially in naturally occurring human prion diseases. The histological abnormalities are not present throughout the central nervous system and the severity of neuronal loss varies according to brain regions. Based on the knowledge that ataxic symptoms and cerebellar neurodegeneration occur frequently in CJD patients, the possible association between neuronal cell loss and the accumulation of various types of PrPSc deposits can be investigated in the cerebellum. The cerebellum provides a good model for studying the statistical correlation between the forms of PrPSc deposition and neurodegeneration, as the severity of neuronal loss is variable from case to case; numerical density of neurones of the granular cell layer can be quantified with precision [10]; PrPSc accumulates following different patterns (e.g. punctate or ‘synaptic’, focal, plaque-like deposits) and congophilic amyloid plaques are formed in some cases.

Sporadic CJD (sCJD) is the most frequent human prion disease with an estimated yearly incidence of approximately 1 to 1.5 case per million population. Various sCJD subtypes have been identified according to a wide spectrum of clinical signs, duration of the clinical phase, electroencephalographic patterns, histological features and biochemical properties of PrPSc. A systematic classification of the disease variants has been proposed, based on (i) the host genotype variability at codon 129 in the PrPSc protein gene (PRNP) that encodes either methione or valine and (ii) distinct physico-chemical subtypes of PrPSc in Western blot analyses, possibly reflecting distinct protein conformations. The classification proposed by Parchi et al. [11–13] is subdivided into six groups depending on the six combinations of the three possible genotypes at codon 129 of PRNP (Met/Met, Met/Val, Val/Val) with two distinct and easily identifiable molecular features of PrPSc conformers. The two PrPSc types are distinguished according to the electrophoretic mobility properties in Western blot analysis after proteinase K treatment, the un-glycosylated forms migrating at approximately 21 kDa and 19 kDa for type 1 and type 2, respectively. Type 2 is also called type 2A with reference to type 2B which is observed in variant CJD, and these two types differ by their ratio of di-glycosylated form. Based on PRNP M129V polymorphism and PrPSc typing, sCJD can therefore be divided into six subtypes: sCJD-MM1, sCJD-MV1, sCJD-VV1, sCJD-MM2, sCJD-MV2 and sCJD-VV2.

In a previous study [10], we have shown that the accumulation of extracellular and diffuse small punctate PrPSc deposits (‘synaptic type’) in the granular cell and molecular layers of the cerebellum is correlated with the loss of cerebellar granule neurones (CGN) in the sCJD-VV2 subtype. Furthermore, we described in the cerebellum of sCJD-VV2 cases a lack of relationship between large focal deposits accumulation and neurodegeneration. This is in accordance with the hypothesis that accumulation and sequestration of neurotoxic misfolded proteins might reduce their pathogenic role [14]. In the present study, we investigated whether these relationships were restricted to sCJD-VV2 or also occurred in other sCJD subtypes. We have examined proven sCJD cases of the six sCJD subtypes.

Materials and methods

Tissue samples selection

Brain tissues were obtained at autopsy from 100 patients with definite sCJD, devoid of mutation or insertion in PRNP coding region. The cases were characterized for clinical and neuropathological data. We studied the six subtypes of sCJD that were determined according to the
molecular type of the abnormal prion protein (PrPSc) and the methionine/valine polymorphism at codon 129 of PRNP (Table 1). There are minor deviations between the age at death and disease duration of some sCJD subtypes in this investigation (Table 1) as compared with published studies [3,12,15–19]; however, all the values of examined cases remain within the ranges of values observed in a large study including 444 sCJD cases [16]. Informed consent for an autopsy, neuropathology and PrP investigation was obtained from patients’ relatives for each case according to the French regulation (L.1232-1 to L.1232-6, Code Santé Publique). A separate informed consent for the genetic investigation of codon 129 polymorphism of PRNP was obtained in accordance with the French Ethics laws (No. 94-653 and No. 94-654). The French National Neuropathology Network for Creutzfeldt–Jakob disease collected the tissue samples. Brain tissue was immersion-fixed in 10% buffered formaldehyde solution. After fixation, tissue-block samples were dissected, placed into cassettes, embedded in paraffin and cut in serial 7-μm-thick sections for histological procedures [20]. Sections from all cases were stained with haematoxylin and eosin, periodic acid-Schiff, Congo red, Bodian silver impregnation coupled to Luxol fast blue and PrP immunohistochemistry. The series of cases that were previously presented in a report published in Journal of Neuropathology and Experimental Neurology [10] were re-examined for the quantification of PrPSc deposition in V/V cases and completed with additional M/M and M/V cases for further PrP immunohistochemical experiments.

### Immunoblot analysis of brain extract

Frozen cerebellum tissue was used to prepare scrapie-associated fibrils as documented [21]. Briefly, a clear lysate from a 5% glucose solution in which tissue had been homogenized [20% (wt/vol)] was treated with proteinase K. After concentration on a 10% sucrose gradient, and resuspension in denaturing buffer, the proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Proteins transferred to nitrocellulose membranes were incubated with a monoclonal antibody directed against amino acid residues 109-112 of human PrP (3F4 clone, 1:50000 dilution; Covance, Emeryville, CA, USA). The signal was detected by horseradish peroxidase-conjugated secondary antibody, enhanced chemiluminescence system and exposure to X-ray film.
The molecular types were classified into PrP<sup>Sc</sup>-type 1 or PrP<sup>Sc</sup>-type 2 according to the pattern of electrophoretic migration [13].

**Molecular genetic analysis**

Genomic DNA was extracted from peripheral blood leucocytes, or frozen brain tissue collected after death. Analysis of PRNP coding sequence was performed on the entire open reading frame [22].

**Immunohistochemistry**

The detection of PrP<sup>Sc</sup> deposits was performed using an automated method (NexES automation, Ventana Medical Systems SA, Illkirch, France) to obtain optimum reproducibility of staining. Sections were placed on coated SuperFrost glass slides (Milan, Bron, France). The antigens were retrieved by hydrated autoclaving (100°C) and 99% formic acid pretreatment before an optimized procedure, which yields a consistent detection of PrP<sup>Sc</sup> with no revelation of cellular isoforms of PrP in control cases [23]. PrP was detected by a specific mouse monoclonal antibody directed against amino acid residues 142-160 of human PrP (12F10 clone, 1:200 dilution; Cayman Chemical, Ann Arbor, MI, USA) and staining was performed using 3,3′-diaminobenzidine as a substrate (iVIEW DAB detection kit; Ventana Medical Systems SA).

**Quantification of numerical density of neurones**

The numerical density of CGN was estimated as documented [10], with the use of a computer-assisted image analysis system in sections of the cortex of the cerebellar hemisphere (Laborlux D microscope equipped with an XY-stage, Leica, Wetzlar, Germany; Z axis micrometric sensor, Avaxex VRZ405 module, Heidenhain, Versailles, France; Exwave colour video camera, Sony, Tokyo, Japan; PC workstation: Mercator software for cell counting within anatomical regions, Pro-Version v1.79, Explora Nova, La Rochelle, France). Sections were coded and analysed in a randomized order. Neurone nuclei were identified by their spherical shape and size in tissue sections stained with 0.1% wt/vol haematoxylin and eosin. Rod shaped and irregularly shaped nuclei with extensive chromatin that were characteristic of microglial cells and very large nuclei corresponding to astrocytes were not recorded. These criteria were validated by the use of CD68 and GFAP immunohistochemistry [10]. The sampling sites for measurements were regularly distributed in the studied sections and counting frames were positioned at approximately 50 μm from the Purkinje cell layer in areas of the granule layer where density of CGN was uniform. The neuronal nuclei were counted within an unbiased counting frame when the top of the nuclei could be observed in the tissue section [24] using an objective with a short depth of focus (×100/1.25 numerical aperture, oil-immersion, Leitz). To obtain cell counts with a coefficient of error between measurements lower than 10%, the quantifications were performed at 20 sites (25 for cases with extensive neuronal loss) in 24 μm × 35 μm counting frames (area, 840 μm<sup>2</sup>). The disector volume of tissue through which the neurones were counted (V<sub>dis</sub>) was calculated from the area of the counting frame (A<sub>frame</sub>) and the measurement of Z-displacement in the tissue width (H, section thickness). The optical disector volume of tissue was given by V<sub>dis</sub> = A<sub>frame</sub> × H. Numerical density of neurones (N<sub>v</sub>) was calculated by N<sub>v</sub> = Q<sup>−</sup>/V<sub>dis</sub>, where the number of counted cells present in the reference section was Q<sup>−</sup>. The numerical density of CGN was not significantly correlated with delay between the time of death and histological fixation or with histological fixation duration. Cases were selected at random within the brain bank collection for each sCJD subtype, the histological state of preservation being a prerequisite, and cases with autolytic/agonal cerebellar granular layer damage were not included in the studied series. Compared to our previous study published in *Journal of Neuropathology and Experimental Neurology* [10], the quantification of numerical density of neurones was performed in 10 additional cases to examine a complete series of cases with both neuronal loss and PrP<sup>Sc</sup> immunohistochemical data.

**Quantification of PrP<sup>Sc</sup> deposition**

The accumulation of immunostained PrP<sup>Sc</sup> deposits was examined for each of the 100 cases in the granular and molecular layers of the cerebellum after randomization of tissue sections. Semi-quantitative grading scales (0–4 scores) were assigned by visual qualitative evaluation for various types of deposits [1,20]: punctate type (diffuse small granular staining, also called synaptic type), 5- to 50-μm-wide deposits (corresponding to focal type, non-amyloid rounded deposits) and plaque-like type (typically larger than 10-μm-wide, rounded deposits with a strongly PrP-immunostained outer ring, more frequent than true...
kuru amyloid plaques which are well detected with haematoxylin and eosin, Congo red and periodic acid-Schiff). A score of 0 represented totally unstained tissue and the scores of 1, 2, 3 and 4 indicated numbers of deposits and staining levels that were low, medium, high or very high respectively.

### Statistical analysis

Data are presented as the mean ± SEM. Parametric and nonparametric tests were applied according to normality and variance of data distribution. SigmaStat statistical program version 3.5 (Systat Software Inc., Point Richmond, CA, USA) was used to analyse the data. The null hypothesis was rejected for an alpha risk equal to 5%.

### Results

#### PrPSc deposition and CGN loss

As PrPSc accumulates in the cerebellum and may play a neurotoxic role on CGN, the PrPSc deposits were scored in the granular cell and molecular layers by using semi-quantitative grading scales to assess punctate deposits, 5- to 50-μm-wide nonamyloid deposits, and plaque-like deposits. For each case, the score for PrP-immunostaining pattern was determined from the observation of many folia in order to limit sampling errors, as variations do often occur in patients with prion diseases. The number of CGN was measured in at least 20 sampling areas to reach a precise estimate of the numerical density of neurones (coefficient of error lower than 10%) in the corresponding sections.

In the 100 sCJD cases, those who presented with clinical signs of cerebellar ataxia had a significantly lower mean numerical density of CGN \[2.3 \times (0.1) \times 10^6 \text{ vs. } 2.8 \times (0.2) \times 10^6 \text{ neurones/mm}^3, P = 0.01, \text{ unpaired two-tailed } t\text{-test} \]. In this large group, the scores for punctate deposits were strongly correlated with the numerical density of CGN (punctate deposits in granular layer: \( r_s = -0.51, P < 0.001; \) punctate deposits in molecular layer: \( r_s = -0.56, P < 0.001 \), Spearman rank order correlation). In contrast, the corresponding correlations for the larger PrPSc deposits scores were not significant, due to differences between subtypes of sCJD patients.

#### Punctate PrPSc deposition, sCJD subtypes and CGN loss

Although the mean numerical density of CGN was significantly higher in sCJD-MM1, -MV1, -VV1, -MM2 and -MV2 subtypes as compared to sCJD-VV2 subtype (Table 1), there were wide variations between individual values within each of these groups. The prevalence of clinical signs of cerebellar ataxia at the end of the disease was high in all groups, except the sCJD-VV1 (Table 1). Cases of the sCJD-MV2 and sCJD-VV2 subtypes that presented cerebellar ataxia at the onset or at the end of illness displayed a loss of CGN as compared to cases with no cerebellar symptoms (neuronal loss was statistically significant in sCJD-VV2 subtype).

The immunohistochemistry of tissue sections with 12F10 anti-PrP monoclonal antibody showed positive patterns of staining of PrPSc deposits as reported in previous studies of sCJD (Figure 1). Semi-quantitative assessment of punctate deposits revealed levels of accumulation that were similar in the granular cell and molecular layers (Table 2) and significantly different between sCJD subtypes (Figure 2). The mean levels were very low in sCJD-VV1, low in sCJD-MM2, highest in sCJD-MV2 and sCJD-VV2, and intermediate in sCJD-MM1 and sCJD-MV1 (Figure 2). Whatever the mean levels, the scores of punctate deposits were negatively correlated with the numerical density of CGN in all sCJD subtypes (except in sCJD-VV1 that included only two cases): the higher the accumulation of punctate PrPSc deposits, the higher the neuronal loss (Figure 3).

#### Large aggregated PrPSc deposition, sCJD subtypes and CGN loss

The 5- to 50-μm-wide and plaque-like types of deposits were found in the granular cell and molecular layers of the cerebellum in sCJD-MV2 and sCJD-VV2 cases (Figure 2). Occasionally, several 5- to 50-μm-wide deposits could be found in a few cases belonging to the other sCJD subtypes, except in sCJD-VV1 where labelling was very low (Figure 1). The mean scores of 5- to 50-μm-wide and plaque-like deposits were highest in sCJD-MV2 (Figure 2).

In the sCJD-VV2 group \((n = 32)\), some cases showed no neuronal loss while others presented a severe depletion and this allowed us to assess the relationship between the scores of 5- to 50-μm-wide deposits and the numerical
Figure 1. Abnormal prion protein (PrP^Sc) accumulation in the cerebellum from sporadic Creutzfeldt–Jakob disease (sCJD) cases. Representative pictures illustrating the various types of PrP^Sc deposits. There were variations in the PrP^Sc pattern of immunostaining between cerebellar folia and between cases within each sCJD subtype (see Figures 3–5). Immunohistochemical labelling for PrP using 12F10 monoclonal antibody reveals sparse and numerous extracellular PrP^Sc deposits which were punctate ('synaptic type') both in the granular cell (gL) and molecular (mL) layers. Large 5- to 50-μm-wide deposits were visualized in sCJD-MV2 and sCJD-VV2 cases and in rare sCJD-MM1, -MV1 and -MM2 cases. Immunoreactive rounded plaque-like deposits with a strongly stained outer ring were observed in sCJD-MV2 and sCJD-VV2 cases. Haematoxylin counterstain. Scale bar represents 50 μm in all panels. gL, granular layer; mL, molecular layer; PcL, Purkinje cells layer.
density of CGN. In this study, owing to the assessment of these deposits in the whole section, we observed that these two variables were positively and significantly correlated, the higher the accumulation of 5- to 50-μm-wide (focal deposits) the higher the neuronal preservation (Figure 4).

The highest coefficient of correlation was observed for the score of 5- to 50-μm-wide deposits found in all sCJD-MV2 cases. The mean scores of 5- to 50-μm-wide deposits were higher in sCJD-MV2 than in sCJD-VV2 cases, with 5-μm-wide deposits located in the molecular layer: \( r_s = +0.42, P < 0.02 \). The scores of punctate deposits were low when those of 5- to 50-μm-wide deposits detected in the molecular layer were high, and correlations between both were statistically significant (punctate in granular layer: \( r_s = -0.41, P < 0.03 \); punctate in molecular layer: \( r_s = -0.49, P < 0.005 \)). As shown in Figure 3, the scores of 5- to 50-μm-wide PrPSc deposits were higher in sCJD-MV2 than in sCJD-VV2 cases, with 5- to 50-μm-wide deposits found in all sCJD-MV2 cases except one (13/14). The scores of 5- to 50-μm-wide deposits were associated with little neuronal loss (Figure 4).

Plaque-like PrPSc deposits were often found in the granular and molecular layers in sCJD-MV2 cases and, at a lower level, in sCJD-VV2 (Figure 2). The mean scores of PrP-immunostained plaque-like deposits differed between the sCJD-MV2 and other sCJD subtypes for deposits located in both the granular and molecular layers (\( P < 0.001 \), Kruskal–Wallis one-way analyses of variance on ranks; Figure 2).

### Table 2. Correlation between the scores of prion protein-immunostained deposits of the punctate type (synaptic type) examined in the granular and molecular layers of the cerebellum

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>sCJD-MM1 (n = 30)</td>
<td>+0.88, ( P &lt; 0.001 )</td>
</tr>
<tr>
<td>sCJD-MV1 (n = 15)</td>
<td>+0.63, ( P = 0.01 )</td>
</tr>
<tr>
<td>sCJD-VV1 (n = 2)</td>
<td>ND</td>
</tr>
<tr>
<td>sCJD-MM2 (n = 7)</td>
<td>+0.84, ( P = 0.01 )</td>
</tr>
<tr>
<td>sCJD-MV2 (n = 14)</td>
<td>+0.63, ( P = 0.01 )</td>
</tr>
<tr>
<td>sCJD-VV2 (n = 32)</td>
<td>+0.90, ( P &lt; 0.001 )</td>
</tr>
</tbody>
</table>

The sCJD-VV1 group included only two cases; therefore, the results of calculations are not presented.

The sCJD sporadic Creutzfeldt–Jakob disease; MM, methionine homozygosity; MV, methionine/valine heterozygosity; VV, valine homozygosity; ND, not determined. (Spearman rank order correlation coefficient).

### Amyloid plaques and CGN loss

In the 100 sCJD cases, those with amyloid plaques detected by periodic acid-Schiff staining in the cerebellum had a significantly higher neuronal preservation than the cases with no occurrence of these plaques \[ 3.1 \ (\pm 0.2) \times 10^6 \text{ vs.} \ 2.3 \ (\pm 0.1) \times 10^6 \text{ neurones/mm}^3, \ P < 0.005, \text{unpaired two-tailed} \ t\text{-test} \]. This result was also observed in the sCJD-MV2 and sCJD-VV2 subtypes [respectively, 3.0 \ (\pm 0.4) \times 10^6 \text{ vs.} \ 2.6 \ (\pm 0.1) \times 10^6, \text{NS}, \text{and} \ 3.1 \ (\pm 0.3) \times 10^6 \text{ vs.} \ 1.7 \ (\pm 0.1) \times 10^6 \text{ neurones/mm}^3, \ P < 0.01, \text{unpaired two-tailed} \ t\text{-test} \]. In addition, the mean duration of disease was longer in sCJD-MV2 cases with amyloid plaques (24 ± 7 vs. 13 ± 2 months, \( P < 0.05 \), unpaired two-tailed \( t\text{-test} \)).

### Discussion

In this study of patients of the various sCJD subtypes, the semi-quantitative assessment of PrPSc accumulation in the granular cell and molecular layers of the cerebellum showed that: (i) the scores of PrPSc deposits of the punctate...
type (synaptic type) are correlated with numerical density of CGN: the higher the score the higher the neuronal loss in patients that are either homozygous or heterozygous at PRNP codon 129, and contain either the molecular isotype 1 or 2 of PrP Sc; (ii) in sCJD-MV2, neuronal loss is not increased despite the highest scores of 5- to 50-μm-wide (focal type) and plaque-like types of PrP Sc deposits; and (iii) in sCJD-VV2, the scores of 5- to 50-μm-wide PrP Sc deposits are positively and significantly correlated with neuronal counts: the higher the score of these large deposits, the higher the neuronal preservation.

This descriptive study was undertaken with the aim of further characterizing the statistical association between the accumulation of PrP Sc deposits and loss of CGN demonstrated in sCJD-VV2 subtype [10]. This subtype was taken as a model as sCJD-VV2 cases frequently present cerebellar ataxia in association with neuronal loss [10,11,25]. In addition to our previous study, we have now shown, in four additional sCJD subtypes that include the most frequent sCJD-MM1 subtype, a sound negative correlation between numerical density of CGN and the scores of PrP Sc punctate deposits that accumulate in the molecular and granule cell layers (Figure 3). The relationship was found in all sCJD subtypes with large numbers of cases. On the whole, our results suggest that the various prion strains that have been associated with sCJD subtypes have some similar neurodegenerative mechanisms [26].

As the punctate PrP Sc deposits have a distribution reminiscent of synaptic terminals, they might be associated with the impairment of synapses in glomeruli of the granular layer and between parallel fibres and Purkinje cell dendrites of the molecular layer, where neurodegeneration process may occur [27]. The precise localization of PrP Sc deposits remains controversial. By colocalization with synaptophysin and synapsin I, fine diffuse small PrP Sc deposits have been shown in presynaptic structures, in the

Figure 2. Abnormal prion protein (PrP Sc) accumulation and numerical density of cerebellar granule neurones. Scores of PrP Sc deposits of the punctate type (‘synaptic type’), 5- to 50-μm-wide (focal type) and plaque-like types in the granular cell and molecular layers of the cerebellum. Cases are arranged according to PRNP M129V polymorphism and PrP Sc molecular type detected in the cerebellum. *P = 0.005, **P < 0.005, ***P < 0.001 (punctate deposits: one-way analysis of variance and pairwise multiple comparison procedures by Holm–Sidak method; plaque-like deposits: Kruskal–Wallis one-way analysis of variance on ranks). gl, granular layer; ml, molecular layer; a.u., arbitrary units.
brain of sCJD cases [28]. In contrast, the results of immuno
gold electron microscopic investigation of Tg(PG14) mice that develop a fatal neurological illness accompanied
by massive apoptosis of CGN demonstrate that punctate
PG14-PrP accumulates on the plasma membranes of
dendrites and axons with no association with pre- or post-
synaptic densities and is associated with degenerative
changes in dendritic structure [29]. Several studies of
infectious prion diseases have shown – in animal models –
that PrPSc accumulates primarily on plasma membranes,
at the level of the neuronal cell body and dendrites
[30–32] and causes severe synaptic dysfunction before
dermic degeneration occurring early in the course of the disease [31,33]. Further inves-
tigation is needed to determine the subcellular localization
and molecular mechanisms that underlie the neurotoxic-
ity of punctate PrPSc deposits in human prion diseases.

In contrast to punctate deposits, large PrPSc deposits
were frequent in sCJD-MV2 and sCJD-VV2 cases, but they
were rare or absent in cases of other sCJD subtypes, as

Figure 3. Correlation between numerical density of cerebellar granule neurones and the scores of punctate extracellular PrPSc deposits (synaptic type) in the cerebellar molecular layer in sporadic Creutzfeldt–Jakob disease (sCJD) subtypes. Cases are arranged according to prion protein genotype at codon 129 and PrPSc molecular type detected in the cerebellum. a, sCJD-MM1 (n = 30); b, sCJD-MV1 (n = 15); c, sCJD-VV1 (n = 2); d, sCJD-MM2 (n = 7); e, sCJD-MV2 (n = 14); f, sCJD-VV2 (n = 32). a.u., arbitrary units; nb, number of neurones; NS, not statistically significant.

Figure 4. Relationship between the numerical density of cerebellar granule neurones and the scores of 5- to 50-μm-wide deposits of PrPSc observed in the molecular layer of sCJD-MV2 (a) and sCJD-VV2 (b) subtypes. a.u., arbitrary units; nb, number of neurones; NS, not statistically significant.
already reported [1,12,20]. Our previous study of the numerical density of CGN in sCJD-VV2 subtype indicates higher neuronal preservation when the number of 5- to 50-μm-wide deposits (focal type) was higher [10]. In the present investigation, based on scores estimating the whole section PrPSc staining, we observed that high scores of PrPSc deposits of 5- to 50-μm-wide and plaque-like types were associated with neuronal preservation in sCJD-MV2 subtype. These results suggest that large aggregates may be at least less neurotoxic than punctate PrPSc deposits, both in sCJD-VV2 and in sCJD-MV2 subtypes. This is an important point to consider when identifying possible therapeutic targets. We also found a definite positive correlation between disease duration and the accumulation of large PrPSc deposits. This is of interest as the properties of the deposit types may have an effect on degenerative processes. The pattern of PrPSc stained deposits in the multiple Gerstmann–Sträussler–Scheinker disease variants is different from that seen in sCJD, but large aggregated PrPSc deposits are also associated with long disease duration [1]. The size and compactness of aggregates has been shown, in transgenic mice models, to differ between forms of misfolded PrP, with a neurotoxic form that would correspond to small, loosely packed oligomers of PrPSc [34]. Mild and diffuse punctate PrP deposits have been reported to accumulate in the molecular and granule cell layers of the cerebellum together with a marked reduction in the number of CGN in transgenic mice overexpressing wild-type PrP [35]. A cryo-immunogold electron microscopic study of the hippocampus from mice infected with the Rocky Mountain Laboratory (RML) strain of prions has shown, in combination with trypsin digestion, that PrPSc is unlikely to act directly on synapses, as most tissue-bound PrPSc was detected in small neurites of the neuropil with no evidence of PrPSc at synaptic junctions, and that the most harmful forms of PrPSc were likely to be soluble small oligomeric forms [36]. Our data favour the hypothesis of neurotoxicity associated with small, beta-rich oligomers rather than large amyloid polymers [35] and suggest that this also may occur in the cerebellum of all sCJD subtypes. This suggests a relationship between the type of deposits and aggregation states of PrP that is not demonstrated in this study. Microdissection of PrP deposits combined to biochemical analyses such as the PrP sedimentation properties after ultracentrifugation in sucrose density gradient could provide some information on the association between the state of aggregation and types of deposits [37].

Our data showing a neuronal preservation in sCJD cases with amyloid plaques (congophilic kuru plaques that can be detected by periodic acid-Schiff staining) and a correlation between the scores of large aggregated PrPSc deposits and disease duration are in agreement with the hypothesis of protective properties in large aggregates which may sequester harmful forms of PrPSc. In the sCJD-VV2 subtype, the scores of 5- to 50-μm-wide deposits and neuronal preservation were positively correlated, together with a significant negative correlation between the scores of 5- to 50-μm deposits and those of punctate deposits, a result in agreement with the hypothesis of a neuroprotective role of large PrPSc deposits. This possible role of large PrPSc deposits can be connected to the detection of clusterin, a heat-shock protein or chaperone molecule, colocalized with PrPSc in deposits and upregulated in the cerebellum of sCJD [38,39]. This glycoprotein is resistant to protease digestion when in plaques but not in punctate deposits, and has been suggested to participate in PrPSc clustering and sequestration, therefore modifying PrPSc toxicity in sCJD [38]. Experiments in a yeast model have shown that the chaperone-dependent assembly of amyloid conformers can facilitate the deposition of
misfolded proteins into an amyloid inclusion and antagonize protein toxicity [40]. Such observations further support the hypothesis of an accumulation and sequestration of misfolded proteins in amyloid plaques that might serve a protective function against toxic species [14].

Taking into account the association between neuronal preservation and the types of PrPSc deposits, we observed similarities between the sCJD-MM1 and sCJD-MV1 subtypes, as well as the sCJD-VV2 and sCJD-MV2 subtypes. In addition, sCJD-VV1 cases presented very different features. These results are consistent with human prion strains that have been recently identified by transmission experiments in mice (‘M1<sup>str</sup>’ strain associated with sCJD-MM1 and sCJD-MV1 subtypes; ‘V2<sup>str</sup>’ strain associated with sCJD-VV2 and sCJD-MV2 subtypes; ‘V1<sup>str</sup>’ strain associated with sCJD-VV1 subtype) [26]. The results of Bishop <i>et al.</i> [26] suggest that the observed correlations may reflect the effects of these prion strains on PrPSc aggregation and the processes of neurodegeneration in the cerebellum.

In conclusion, the investigation of all sCJD subtypes in this study demonstrates a sound relationship between the loss of CGN and the level of accumulation of PrPSc deposits of the punctate type in the cerebellum. The data support a high neurotoxicity connected with sparse small deposits whatever the genotype of the patients at codon 129 of PRNP and whatever the molecular isotype of PrPSc. In contrast, there was no similar association between large PrPSc deposits accumulation and neuronal loss in both sCJD-MV2 and sCJD-VV2 subtypes. The observations reported here suggest that PrPSc located in large aggregates would be associated with lower neurotoxicity than PrPSc located in small deposits and are in accordance with the hypothesis of a possible protective role of large aggregates. A better knowledge of toxic properties of PrPSc that accumulate in the human brain may help to identify targets for neuroprotective strategies in CJD.

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