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Neuronal Apoptosis in Fatal Familial Insomnia

Anne Dorandeu[1], Laure Wingertsmann[2], Fabrice Chrétien[2], Marie-Bernadette Delisle[3], Claude Vital[4], Piero Parchi[5], Pasquale Montagna[6], Elio Lugaresi[6], James W. Ironside[7], Herbert Budka[8], Pierluigi Gambetti[5], Françoise Gray[1,2]

[1] Laboratoire de Neuropathologie, Faculté de Médecine Paris-Ouest, Garches, France.
[2] Laboratoire d'Histologie, Embryologie et Cytogénétique, Faculté de Médecine Pitié-Salpêtrière, Paris, France.
[3] Laboratoire d'Anatomie Pathologique, Faculté de Médecine Rangueil, Toulouse, France.
[4] Laboratoire de Neuropathologie, Université Victor Ségalen, Bordeaux, France.
[5] Division of Neuropathology, Institute of Pathology, Case Western University, Cleveland, Ohio, USA.
[6] Institute of Clinical Neurology, University of Bologna, Bologna, Italy.
[7] Neuropathology Laboratory, Department of Pathology, the University of Edinburgh, Edinburgh, UK.
[8] Institute of Neurology, University of Vienna, Vienna, Austria.

The possibility that neuronal loss in prion diseases occurs through an apoptotic process has been postulated and is consistent with the lack of inflammation in these disorders. In order to test this hypothesis in FFI, in which neuronal loss is the predominant neuropathological feature, we examined samples of thalamus, basal ganglia, cerebral cortex, cerebellum and medulla from 10 subjects with FFI. All the patients had the characteristic 178 N mutation of the PrP gene. Eight subjects were homozygous methionine/methionine at codon 129 and 2 were heterozygous methionine/valine. Apoptotic neurons were identified by *in situ* end labelling in all the FFI cases and in none of the controls. They were mostly found in damaged regions and their presence and abundance seemed to correlate closely with the neuronal loss. They were particularly abundant in the thalamus and medullary olives. In heterozygous cases who had a longer disease duration and more widespread cerebral changes, apoptotic neurons were also found in the neocortex and striatum. The abundance of apoptotic neurons also correlated well with microglial activation as demonstrated by the expression of major histocompatibility complex class II antigens. PrP^{res} immunostaining was almost invariably negative, consistent with previous data showing the lack of obvious correlation between neuronal loss and PrP^{res} deposits in prion diseases.

Corresponding author:
Professor Françoise Gray, Laboratoire de Neuropathologie, Faculté de Médecine Paris-Ouest, F-92380 Garches, France. Tel. : (33) 01 47 10 76 92; Fax: (33) 01 47 10 76 93; E-mail: francoise.gray@rpc.ap-hop-paris.fr

Introduction

Neuronal loss, together with spongiosis and astrocytosis, is a salient feature of human prion diseases (23). However, its causes and mechanisms, particularly its relationships with the accumulation of the pathogenic, protease resistant isoform (PrP^{res}) of the cellular prion protein (PrP^{c}), are still unclear. The possibility that neuronal loss in prion diseases occurs through a process of programmed cell death has been postulated. Programmed cell death (PCD) is a physiological form of the cell-suicide process, essential in the normal development, maturation and turnover of tissues. However, abnormal induction of a cell death programme may occur in pathological conditions. PCD is an active process, requiring activation signals, signal transduction and, in most instances, gene expression and protein synthesis in the dying cell. It is therefore regulated by signals provided by the local environment. Unlike necrosis, which is a passive, pathological form of cell death, PCD can be induced or suppressed in most instances by the withdrawal or addition of defined activation signals (7, 31). Apoptosis, the phenotype of cell suicide, differs from necrosis morphologically and biochemically. Morpho-logically it is characterized by shrinkage of the cell and the nucleus, condensation and fragmentation of the nuclear chromatin, loss of the nuclear membrane integrity, maintenance of organelles and plasma membrane integrity despite membrane blebbing, and the segmentation of the cell into apoptotic bodies that are rapidly ingested by neighbouring phagocytic cells. Unlike necrosis, in which cell swelling and rupture of the cell membrane lead to release of proteases and intracellular toxic enzymes causing an inflammatory reaction, PCD does not induce inflammation in the affected tissue (7, 29). Biochemically, apoptosis is characterized the specific endonuclease-mediated internucleosomal

case number	age	sex	disease duration (months)	codon 129	reference
1	38	M	12	Met/Met	Colombier et al. [9]
2	63	F	10	Met/Met	Collinge et al. [8] Case PDG 1385 Will et al. (this issue) case 1
3	38	F	14	Met/Met	Will et al. (this issue) case 2
4	25	M	13	Met/Met	Budka et al. [6] Case 1
5	20	F	10	Met/Met	Budka et al. [6] Case 2
6	58	F	8	Met/Met	Budka et al. [6] Case 3
7	53	M	8	Met/Met	Medori et al. [24] Case IV 26 FFI 2
8	54	F	11	Met/Met	
9	56	F	33	Met/Val	Manetto et al. [22] Case IV 16-FFI 1
10	35	F	25	Met/Val	Manetto et al. [22] Case V 58-FFI 1

Table 1.

fragmentation into regular subunits multiple of an oligonucleosome length unit of 180 base pairs (32).

The hypothesis that, in prion diseases, neuronal loss occurs through an apoptotic process is consistent with the almost complete absence of inflammatory reaction in their pathology. It is also supported by experimental studies. Apoptosis of neurons has been identified, *in vivo*, using *in situ* end labelling (ISEL) and electron microscopy in scrapie infected mice (16). Moreover, the 106-126 synthetic peptide homologous to PrP has been shown to be neurotoxic and induce apoptosis of rat hippocampal neurons in cultures (12). These findings have been recently supported, in human, by a preliminary ISEL study in sporadic, familial, and iatrogenic Creutzfeldt-Jakob disease (CJD) (17).

We looked for neuronal apoptosis in fatal familial insomnia (FFI) and compared our findings with those in CJD, since FFI differs from other prion diseases in that neuronal loss and gliosis in the medial thalamus are the predominant neuropathological feature (13, 21, 22, 24) whereas spongiosis is moderate or absent. Moreover, although the presence of PrP^{res} has been demonstrated by immunoblotting in restricted brain areas (27), its amount is usually small and it is generally undetectable by immunocytochemistry.

Material and Methods

In the framework of 2 European Union Concerted Actions on human transmissible spongiform encephalopathies (BMH4-CT97-2034) and FFI (BMH4-CT96-0856), brain samples from 10 patients who died from FFI were collected. They included one case from France (9) (case 1), 2 unrelated patients from UK (cases 2 & 3) one of whom had been successfully transmitted to transgenic mice expressing human PrP (8), 3 members of the recently described Austrian family (6) (cases 4-6), and 4 cases from 3 different Italian kindreds (22, 24, 27) (cases 7-10). All the patients had the typical Asn mutation at codon 178 of the PrP gene. Eight patients (cases 1-8) were homozygous methionine/methionine at codon 129, and 2 patients were heterozygous methionine/valine (cases 9 and 10). Clinical, epidemiological, genetic and bibliographic informations concerning these 10 cases are summarized in table 1. Ten age and sex matched individuals who died accidentally but with no brain involvement, collected in Forensic Medicine, were examined according to the same protocole and served as controls.

In each case we examined samples of thalamus at the level of the anterior nucleus, striatum, at least one sample of cerebral neocortex and Ammon's horn, cerebellum, and medulla. Sections from formalin fixed, formic acid post fixed, paraffin embedded specimens were stained with H&E. Immunocytochemistry to demonstrate prion protein deposits was performed according to the protocol of the CJD Surveillance Unit, Edinburgh (4) with microwave substitution of the autoclaving pretreatment, using the 3F4 antibody (Senetek). Immunocytochemistry to identify major histocompatibility (MHC) class II antigens and interleukin (IL)-1β expression was performed after microwave pretreatment, by an immunoalkaline phosphatase (APAAP) method using a monoclonal anti-human MHC class II (HLA-DR) antibody (Dako) and a polyclonal anti-human IL-1β antibody (Genzyme, UK). *In situ* end labelling was performed using the Apoptag kit (Oncor) modified as previously described (1).

The presence of apoptotic neurons was evaluated semiquantitatively by two different neuropathologists (AD & FG) and subsequently reviewed jointly, as previously described (1); briefly it was scored 0 when no apoptotic neurons were found, (+) when only occasional isolated apoptotic neurons were seen, (++) when occasional nests of apoptotic neurons were observed

and (+++) when apoptotic neurons were frequent. Endothelial cells which have a quick turn over and therefore often undergo apoptosis, served as positive internal controls. The intensity of PrPsc, HLA-DR and IL-1β expression was also evaluated semiquantatively and scored 0: absent, (+): mild, (++): marked and (+++): intense.

Results

Apoptotic neurons were identified in all the FFI cases (table 2) and in none of the controls. Positive *in situ* end labelling was frequently but not invariably associated with morphological changes characteristic of apoptosis with pyknotic nuclei and shrunken cytoplasm. Apoptotic neurons were mostly found in damaged regions and their topography and abundance seemed to correlate closely with those of the lesions. Microglial activation as demonstrated by expression of HLA-DR was also closely related in distribution and severity to neuronal apoptosis (table 2). Expression of IL-1β was only found in areas in which HLA-DR expression was intense. It was always discrete, never exceeding (+), mainly identified in the cytoplasm of occasional macrophages and more rarely in process bearing microglial cells. PrP imunostaining was invariably negative with the exception of one Austrian case (case 6) (6) in which weak PrP positivity was identified in the molecular layer of the cerebellar cortex.

In patients homozygous for methionine at codon 129 of the PrP gene, the predominant changes included neuronal loss and gliosis in the thalamus, particularly in the anterior nucleus. Involvement of the medullary olives with neuronal loss, frequent vacuolated neurons and gliosis, was also usually present. Involvement of the cerebral cortex and striatum was generally inconspicuous restricted to inconstant rare spongiform change, and the cerebellum was unremarkable. Apoptotic neurons were most numerous in the anterior nucleus of the thalamus involving, in some cases, most of the remaining neurons (Fig. 1A). They were not limited to the anterior nucleus and usually extended to other thalamic nuclei in which neuronal loss was incomplete. Parallelly, microglial activation was severe in the thalamus (Fig. 1B), particularly in the anterior nucleus and medial thalamus, but it involved also, to a lesser extent, the other thalamic nuclei. In contrast, Il-β expression was constantly weak, even in regions with marked HLA-DR expression. Apoptotic neurons were constantly identified in the medullary olives (Fig. 1C) and in the surrounding medullary nuclei. High expression of HLA-DR was also always found in the medulla. It was obvi-

n° case	thalamus			medulla			striatum			cortex			cerebellum		
	NP	HLADR	apopt	NP	HLADR	apopt	NP	HLADR	apopt	NP	HLADR	apopt	NP	HLADR	apopt
1	gl +++	+++	+++	gl ++	+++	+	sp +	+	+	sp ±	-	+	-	-	-
2	gl ++	++	++	vac	++	++	sp +	+	+	-	-	+	-	-	-
3	gl ++	+	++	vac	++	+	sp +	+	+	-	-	-	-	-	-
4	gl +++	++	++	gl ++	++	++	-	-	-	-	-	-	-	-	-
5	gl +++	+++	+++	gl +++	+++	+++	-	-	-	sp ±	-	+	-	-	-
6	gl ++	++	++	gl ++	++	++	-	-	-	sp +	++(AH)	++(AH)	-	+	+
7	gl +++	+++	++	gl +	++	+	sp +	-	+	-	-	-	-	-	-
	19			**14**			**5**			na			na		
8	gl +++	++	++	vac	+++	+++	sp +	+	+	sp +	+	++	-	-	-
9	gl ++	++	++	n.a.	n.a.	n.a.	gl +	+	+	sp ++	++	++	n.a.	n.a.	n.a.
	26			**6**			**24**			**100**			**18**		
10	gl +	+	+	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	sp +	++	+	gl +(DN)	++(DN)	+
	21			**10**			**34**			**63**			**5**		

NP= neuropathology; apopt = apoptotic neurons; gl = gliosis; sp = spongiosis; vac = vacuolated neurons; n.a. = not available;
AH = Ammon's horn; DN = dentate nucleus
In cases 7, 9 and 10, the amount of PrP res evaluated by immunoblot by Parchi et al. [27] has been indicated in bold numbers under the neuropathology

Table 2.

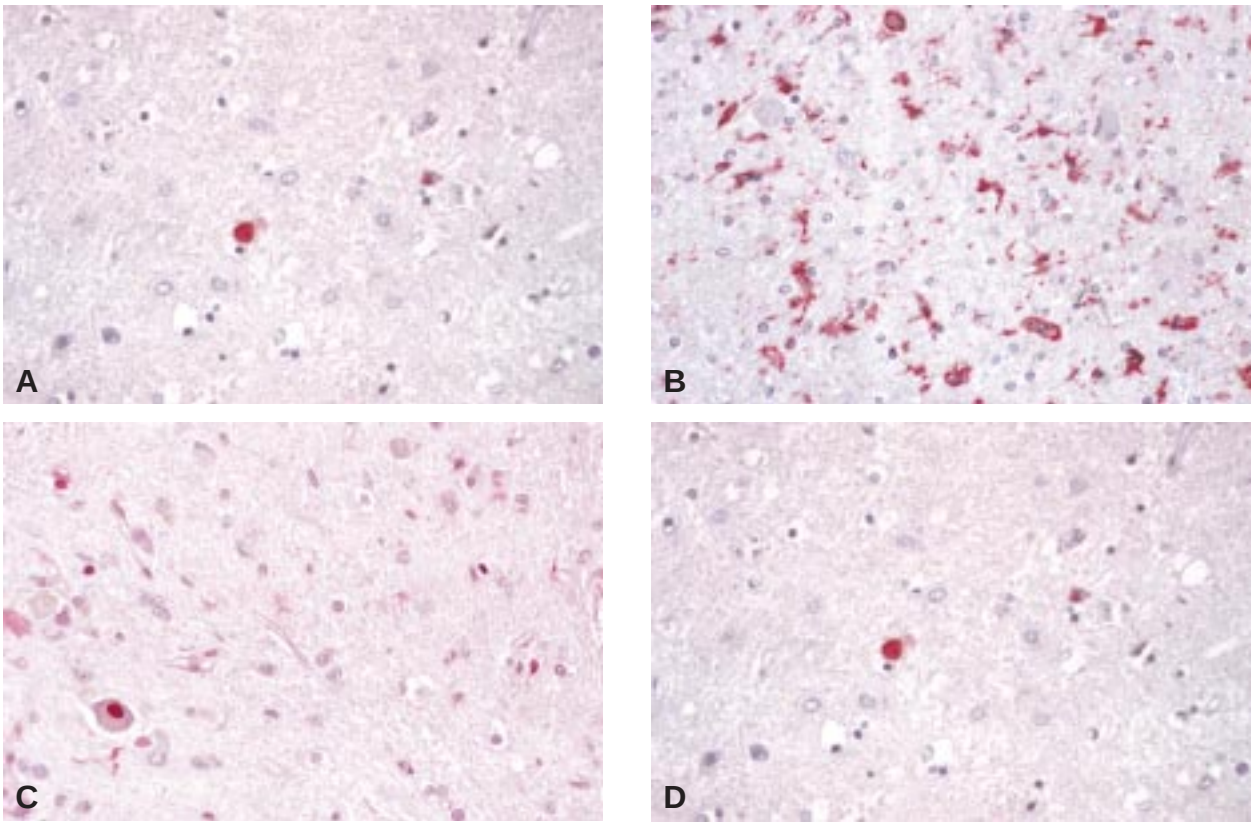

Figure 1. Lesions in patients homozygous Met/Met at codon 129 of the PrP gene. **A**) case 7, anterior nucleus of the thalamus, ISEL stains positively the only remaining neuron, x 400. **B**) case 1, anterior nucleus of the thalamus, HLA-DR immunostaining shows marked microglial activation, APAAP x 200. **C**) case 6, medullary olive, ISEL stains positively a neuron with a pyknotic nucleus, x400. **D**) case 8, cerebral neocortex, ISEL stains positively one neuron in the vicinity of mild spongiform change, x400.

ous, often multifocal, in the olives, but was often more intense in the dorsal medullary nuclei. In the cortex and striatum, only a few labelled neurons were occasionally identified, mostly in the vicinity of the rare spongiform change (Fig. 1D) and HLA-DR expression was usually normal. Occasional positive granular neurons and microglial activation were found in the cerebellum in one case (case 6), interestingly the only one in which discrete PrP expression was observed in the cerebellum (6).

As already stressed (13), different lesions were seen in the brains of the 2 subjects who were heterozygous methionine/valine at codon 129 of the PrP gene and had a longer disease duration. Neuronal loss and gliosis in the thalamus were less severe than in homozygous patients, although apoptotic neurons were frequent and microglial activation was marked. In contrast, changes in the neocortex were more severe and included spongiosis, frequent apoptotic neurons (Fig. 2A) and high HLA-DR expression (Fig. 2B). Il-β expression was present but weak (Fig. 2C). Examination of the striatum performed in case 9 only, revealed prominent microglial activation associated with marked gliosis and a few apoptotic neurons (Fig. 2E). In this area apoptotic microglial cells were also identified.

Discussion

Apoptosis of neurons was identified using *in situ* end labelling in all our 10 cases with fatal familial insomnia and not in the controls, supporting the view that programmed cell death is a feature of human prion diseases (17) and may contribute, at least partly, to the neuronal loss which is a major feature of these conditions. Apoptotic neurons were mostly found in affected brain regions and their distribution and abundance correlated closely with neuronal loss. They were invariably found in the thalamus and medullary nuclei. The two subjects who were heterozygous methionine/valine at codon 129

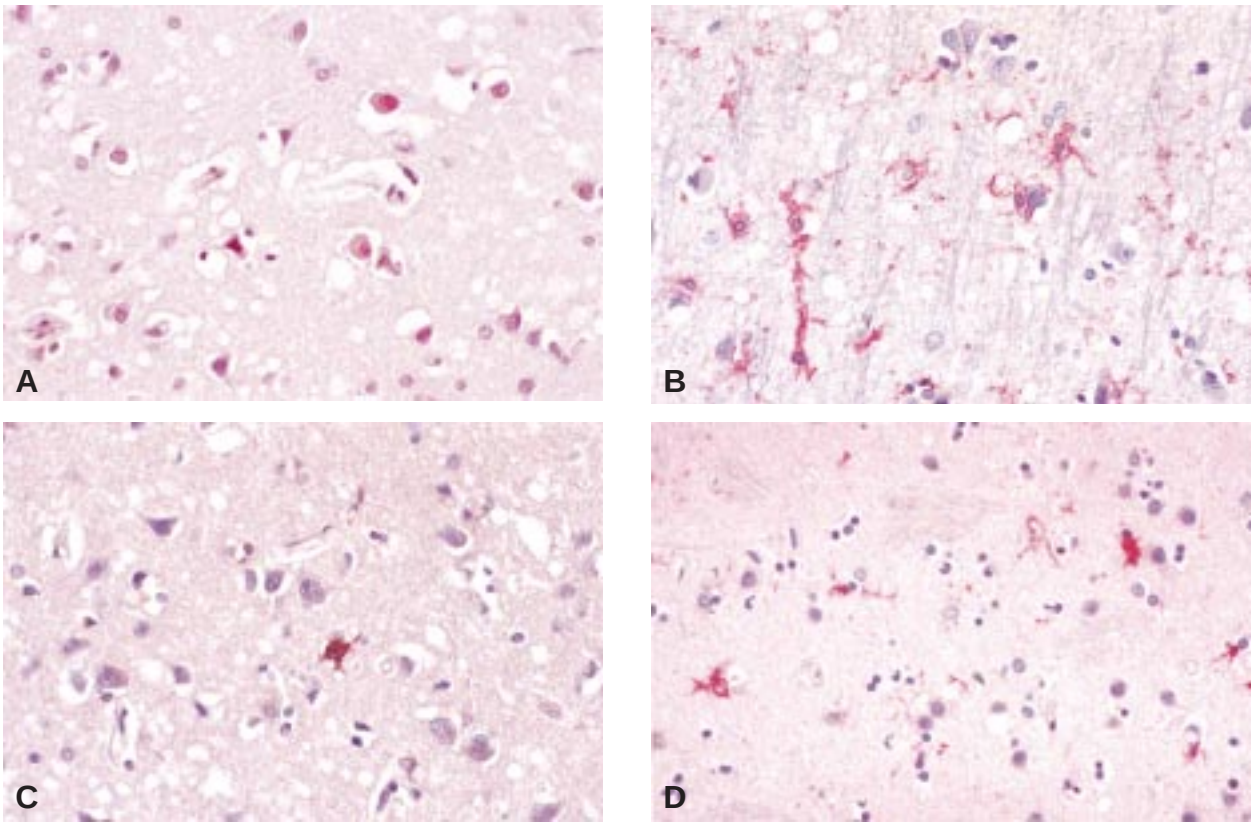

Figure 2. Lesions in patients heterozygous Met/Val at codon 129 of the PrP gene. **A**) case 9, cerebral neocortex, ISEL stains positively several neurons some of which show morphological features characteristic of apoptosis within marked spongiform change, x 400. **B**) case 9, cerebral neocortex same area as 2b, HLA-DR immunostaining shows marked microglial activation, APAAP x400. **C**) case 9, cerebral neocortex same area as 2b and 2c, IL-1b immunostaining shows weak expression in a the cytoplasm of a process bearing microglial cell, APAAP x 400. **D**) case 9, striatum, ISEL stains positively one neuron with a pyknotic nucleus and a shrunken cytoplasm, and one process bearing microglial cell, x 400.

of the PrP gene and had a longer disease duration with more diffuse cerebral lesions (13) had more widespead apoptotic neurons also detected in the cerebral cortex and striatum.

Microglial activation, as demonstrated by expression of major histocompatibility class II antigens, was also closely related to the other neuropathological changes in distribution and severity, suggesting that it is, together with the astrocytic reaction, a component of the gliosis which is a main neuropathological characteristic of FFI. This is consistent with previous studies demonstrating that microglial activation is a feature of human spongiform encephalopathies (25). The close relationship, both topographic and quantitative, between microglial activation and neuronal apoptosis suggests two possible mechanisms which are not mutually exclusive. First, the microglial reaction may just be the consequence of neuronal apoptosis or neuronal damage. Transformation of microglia into brain macrophages has been shown to occur in response to several types of injury, including anterograde and retrograde axonal or neuronal lesions (14, 15) and, it may also be directed to the ingestion of apoptotic neurons (29). Alternatively, microglial activation may play a causative role in neuronal apoptosis. The differentiation of microglia into brain macrophages is accompanied by the release of cytotoxic mediators such as free oxygen radicals, cytokines or nitric oxide (NO) (3). We investigated specifically IL-1β as it has been shown that this cytokine may activate the immunologic form of nitric oxide synthase (11) and that chronic NO exposure produces apoptosis of cells (5). However, although IL-1β expression was constantly associated with HLA-DR expression, it was unvariably much weaker. This differs from HIV encephalitis in which production of IL-1β has been shown to be associated with microglial activation and in which, in our experience, the expression of IL-1β is superposable on that of HLA-DR (2). This discrepancy may be explained

by the presence of an inflammatory reaction that may enhance IL-1β expression in HIV encephalitis, whereas it is absent in FFI.

The presence of frequent apoptotic neurons, comparable in number to what we observed in other prion diseases (17), in the absence of immunocytochemically identifiable PrP^{res} deposits is consistent with previous observation that neuronal damage in prion diseases does not parallel PrP^{res} deposition. Indeed, in the only case in whom PrP^{res} deposits were identified in the cerebellum, these involved the molecular layer, whereas the only occasional apoptotic neurons demonstrated by ISEL, in this area, were in the granular layer. The presence of PrP^{res} was detected by immunoblot in the grey matter of all nine FFI cases examined by Parchi *et al*. (27); however, as underlined by the authors, although PrP^{res} showed a selective pattern of distribution, there was no exact correlation between its amount and the severity of histological changes, and its distribution was more widespread than the histopathological changes. Similarly, as illustrated in table 2 where we have indicated the amount of PrP^{res} evaluated by Parchi *et al*. (27) below the histopathological changes, there was no close correlation between the amount of PrP^{res} and the intensity of neuronal apoptosis in 3 of these 9 cases that we could examine (cases 7, 9 &10).

The mechanism of neuronal apoptosis in FFI and its relationship with PrP^{res} production is speculative. The dissociation of neuronal damage from PrP^{res} deposition may support models of neuropathogenesis based on loss of function of PrP, such as withdrawal of defined activation signals inducing PCD, rather than neurotoxicity (6, 8). Other studies suggest that PrP^{res} is neurotoxic and that the dissociation between neuronal damage and the amount of protein only reflects the variability in different brain regions of the timing and rate of accumulation of PrP^{res}, and selective neuronal vulnerability (10, 27). It has been postulated that PrP^{res} may be neurotoxic through an excitotoxic glutamate-mediated mechanism, since PrP^{res} associated neuronal death may be blocked by antagonists of the NMDA receptor complex (26), and glutamate-mediated neurotoxicity has been postulated to act through a PCD mechanism (20). Finally, neuronal apoptosis might be an indirect consequence of PrP^{res} deposition. Recent experimental studies in scrapie infected mice concluded that pre-amyloid PrP release and accumulation is not invariably toxic either to the neuron releasing PrP^{res} or to the neuropil in which it is released; however, axon terminal degeneration and dendritic spine loss in some neuroanatomical areas might be indicative of specific PrP^{res} toxicity, and may be the cause of neurological dysfunction (19). It may therefore be postulated that PrP^{res}-induced dendritic or axonal damage can contribute to neuronal apoptosis either due to deafferentation (28) or to retrograde neuronal degeneration (18). Such a mechanism would be consistent with the observation in our case 6, as in observations of familial CJD with octapeptide repeat insertions (17, 30), of PrP^{res} deposits in the cerebellar molecular layer and neuronal apoptosis in the granular layer.

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