Chapter 2

NEUROPATHOLOGY OF TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES (PRION DISEASES)

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2.1. Introduction

The transmissible spongiform encephalopathies (TSEs) or prion diseases are a group of neurodegenerative disorders which include kuru[1], Creutzfeldt-Jakob disease (CJD)[2], variant Creutzfeldt-Jakob disease (vCJD), Gerstmann-Sträussler-Scheinker (GSS) disease[3], and fatal familial insomnia[4] in man, natural scrapie in sheep, goats and mufflons, transmissible mink encephalopathy in ranch-reared mink[5], chronic wasting disease of mule deer and elk in the USA[6] and Canada, bovine spongiform encephalopathy (BSE) or "mad cow disease"[7] and its analogues in several exotic species of antelopes and wild felids in zoological gardens and feline spongiform encephalopathy in domestic cats.

These disorders are caused by a still not completely understood pathogen variously referred to as a "prion" (predominantly) or a slow, unconventional or atypical virus, or "virino" (rarely). Despite wide acceptance for the prion theory, these names still reflect different views about the true molecular structure of the pathogen and, by the same token, our ignorance of its nature. Those who prefer to view this pathogen as composed "predominantly or exclusively" of a pathologically folded protein

(PrP^{Sc}; Sc from scrapie or PrP^{d}; d from disease), use the term "prion"; hence the term "prion diseases".

The "virino" hypothesis suggests that the pathogen is a molecular chimera composed of a still-to-be-discovered nucleic acid and a shell-protein which is host-encoded (perhaps PrP^{d}). The virus hypothesis simply suggests that the pathogen is a yet-to-be-identified unconventional virus. The "unified theory" of Weissmann[8], not unlike the virino theory, suggests that the agent is a molecular chimera of the misfolded protein that confers infectivity and an unidentified oligonucleotide that specifies strain characteristics.

2.2. Nomenclature

The nomenclature of PrP species is confusing. PrP^{c} is a normal cellular isoform. PrP^{Sc} (PrP^{res} or PrP^{d}, from disease) is a pathological misfolded protein. PrP^{Sc} is operationally defined as resistant to proteinase K (PK) and insoluble in denaturing detergent; however, in some diseases, pathological isoform of PrP is not PK resistant[9]. Thus, we prefer to use the neutral term PrP^{d} which denotes that misfolded species of PrP which is disease-associated; PK-resistant or not. PrP 27-30 is a proteolytic cleavage product of PrP^{d} which is sometimes referred to as PrP^{res} (res from resistant) when generated following incomplete proteolytic digestion in Western blotting.

2.3. PrP, its gene, the ''prion" hypothesis

PrP^{c} is a highly conserved sialoglycoprotein encoded by a cellular gene mapped to chromosome 20 in man and 2 in mouse[10]. The gene is ubiquitous; it has been cloned in numerous mammalian species included marsupials and there are analogues of this gene in birds, reptiles, amphibians, and recently fish; those in Drosophila and nematodes appeared to be cloning artefacts. PrP 27-30 was first discovered as a protein co-purifying with infectivity in extracts derived from brains infected with the 263K strain of scrapie agent which led to the conclusion that PrP is a part of infectivity.

The "prion" hypothesis, which is deeply rooted in this association between PrP and infectivity, was formulated by Stanley B. Prusiner in 1982[11]. The hypothesis postulated that the scrapie agent was a proteinaceous infectious particle, because infectivity was dependent on protein but resistant to methods known to inactivate nucleic acids. A similar proposal had been presented a decade earlier by many investigators who all developed the earlier suggestion based on irradiation studies, that scrapie agent was devoid of disease-specific nucleic acid[12].

Like many amyloid proteins, PrP 27-30 is a proteolytic cleavage product of a precursor protein, PrP 33-35^{d}. However, PrP 33–35^{d} is not the *primary* product of the cellular gene. It has an amino acid sequence and posttranslational modifications (like glycosylation and the attachment of GPI, glycophospholipid inositol anchor) identical to those of PrP 33–35^{c}, but strikingly different physicochemical features; in particular, PrP^{c} is completely degraded by a limited proteolysis but PrP^{d} is only partially degraded, yielding a core protein (PrP 27–30) which may be visualised by electron microscopy as scrapie-associated fibrils (SAF), better known as prion rods[13]. To become PrP^{d}, PrP^{c} must be first transported to the cell surface and then through the endosomal-lysosomal pathway.

PrP has several interesting features. As already mentioned, PrP is a glycoprotein with two Asn-glycosylation sites; thus, PrP may exist as deglycosylated, monoglycosylated and di-glycosylated isoforms of different electrophoretic mobilities and glycoforms[14]. The various combinations of glycosylation and codon 129 genotype (see later) correlate to some degree with the phenotypic expression of human TSE. In particular, a distinctive glycosylation pattern is uniquely present in both BSE and vCJD[14,15]. Although glycosylation patterns breed true—i.e., they are retained in passage[14]—changes in electrophoretic mobility may occur in the presence of metal ions[14], and more than one pattern may occur in different regions of the same brain, or brain and peripheral organs in the same patient.

PRNP gene in humans consists of two exons and the whole ORF is confined to the second exon[16]. The polymorphism at codon 129 merits special comment. Codon 129 encodes Met in ca 60% and Val in 40% of alleles in the normal Caucasian population. However, in all forms of CJD, there is marked over-representation of homozygotes over heterozygotes. The codon 129 polymorphism may also exert a modifying effect on the phenotypic expression of a given *PRNP* mutation.

The situation in kuru is particularly interesting. The practice of cannibalism underlying the kuru epidemic created a selective force on the prion protein genotype. As in CJD, homozygosity at codon 129 ($129^{Met\ Met}$ or $129^{Val\ Val}$) is overrepresented in kuru. However, Mead *et al.*[17] found that among Fore women over fifty years of age, there is a remarkable overrepresentation of heterozygosity ($129^{Met\ Val}$) at codon 129, which is consistent with the interpretation that $129^{Vam\ Met}$ makes an individual resistant to TSE agents and that such a resistance was selected by cannibalistic rites. Because of this $129^{Met\ Val}$ heterozygote advantage, it has been suggested that the heterozygous genotype at codon 129 has been sustained by a widespread ancient practice of human cannibalism.

2.4. Classifications

From early days, CJD (the name as Jakob-Creutzfeldt disease was coined by Spielmeyer in 1922) has been sub-classified into several forms. For instance, Daniel[18] singled out the classical cortico-striato-spinal (Jakob) type; Heindenhain type (characterized by cortical blindness due to severe involvement of the occipital lobes); diffuse type (dementia with pyramidal and extrapyramidal signs and symptoms) and ataxic type[19]. Siedler and Malamud[20] discriminated cortical, cortico-striatal, cortico-striato-cerebellar, cortico-spinal and cortico-nigral type. In the literature, CJD exists under more than 50 different names and many of these do not represent CJD in a modern sense. The discrimination of all these variants is merely of historical interest but recent molecular studies substantiated the existence of certain defined phenotypes.

PrP^{res} (after limited proteolytic digestion) may exist as 21 kDa (type 1) and 19 kDa (type 2) isoforms which coupled with the status of codon 129 of the *PRNP* gene underlie the existence of 7 molecular variants—MM1, MV1, MM2- cortical, MM2—thalamic, MV2, VV1 and VV2. These variants differ both clinically and neuropathologically[21]. Type MM1corresponds to classical sporadic CJD with changes in the cerebral cortex, striatum, thalamus and the cerebellum; PrP^{d} accumulates mostly as synaptic deposits. This type comprises approximately 70% of sCJD cases. Second, most common type, VV2 comprises approximately 15% of all sCJD cases. Changes are confined to the limbic system, striatum, the cerebellum, thalamus and hypothalamus and several brain stem nuclei. The involvement of the cerebral cortex depends on the duration of illness; those cases of short duration may exhibit minimal cortical changes, spongiform change demonstrates laminar distribution while PrP^{d} accumulates as plaque-like, perineuronal and synaptic deposits. MV2 type (approximately 8%) is reminiscent of VV2 type—spongiform change is confined to the subcortical structures while PrP^{d} expression is mostly plaque-like. In contrast to MV2 type, in VV2 type—"true" (i.e., congophilic and visible in a routine H & E stain) plaques predominates. MM2 type is further sub-classified into MM2-thalamic, which corresponds to FFI and FSI cases and MM2-cortical, similar to MM1 type, from which differs by limited cerebellum involvement and larger (coarse) vacuoles. VV1 is very rare ($<$1% of all sCJD)—changes are limited to cerebellar cortex and the striatum while other structures, including the cerebellum are barely involved.

A more refined approach was used by Collinge *et al.*[14,22] who exploited the size of PrP^{d} fragments following limited PK digestion and the relative abundance of mono-, di- and deglycosylated glycoforms. This

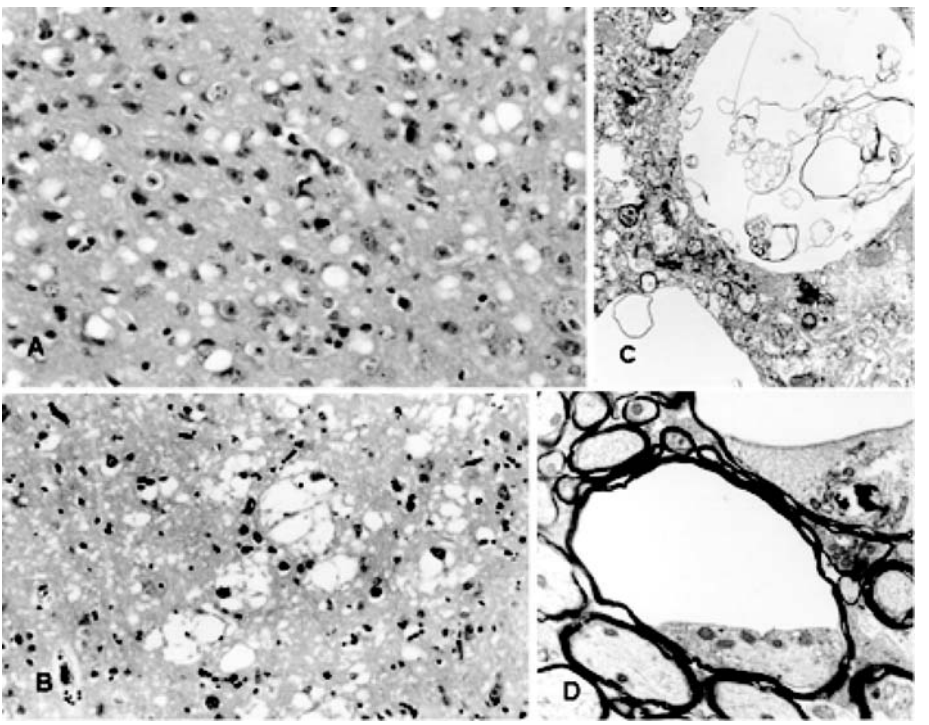
approach discriminated PrP^{d} types 1–4 and 6; type 5 exists in vCJD-infected transgenic mice but not in humans. All type 1 cases are homozygous for Met at codon 129 of the *PRNP* gene; type 2 may exist coupled with every status of the codon 129; type 3 is associated with at least one 129^{Val} allele with the exception of a single CJD cases homozygous for Met at codon 129. Type 4, characterized by predominance of diglycosylated glycoform, is unique for vCJD and BSE[15]. These types differ neuropathologically as well as clinically. Type 1 cases demonstrated widespread spongiform change in the cerebral cortex, mild changes in the basal ganglia, cerebellum and brainstem but no spongiform degeneration in the hippocampus. In type 2 homozygous for 129^{Met} cases, basal ganglia are moderately affected while in heterozygous type 2 cases or type 2 homozygous for 129^{Val}, the basal ganglia are involved severely. Type 3 MV cases are characterized by the presence of kuru plaques already seen on routine H & E preparation.

The translation of the Collinge scheme into the Gambetti is not straightforward, probably due to technical differences in the methodology for Western blots. Furthermore, chelation of metal ions performed prion to PK digestion interconverts both type 1 and 2 MM PrP fragments into so called 2^{-} PrP[23]. Having said this, the Collinge's type 1 MM, type 2 MM, type 3 VV, type 2 MV and type 3 MV are similar to the Gambetti's type MM1, MM2-cortical, VV2, MV1 and MV2, respectively. Thus, it seems that the Collinge sub-classification and the Gambetti sub-classification are, basically, interconvertible. This notion has been supported by recent work which indicates that alterations in electrophoretic mobility can be markedly influenced by pH variations in the brain tissue homogenate. When pH is controlled, it appears that two major subgroups of PrP^{res} can be identified in terms of electrophoretic mobility of the unglycosylated band, corresponding to the types 1 and 2 of the Gambetti *et al.* classification.

2.5. Classical Neuropathology

Creutzfeldt in 1920[24] described one case of a novel neurodegenerative conditions and Jakob described sequentially 5 cases[25,26]. Four Jakob's cases, still on files at the University of Hamburg, were reexamined by Masters and Gajdusek[27] who confirmed that 2 Jakob's cases fulfill modern criteria of CJD while remaining 2 cases represent other not well defined neurological conditions. Of special interest is one of Jakob's cases with amyotrophy which initiated a long-lasting confusion of "amyotrophic type of CJD" that appear to be merely amyotrophic lateral sclerosis with dementia and which is not transmissible[28]. Neuropathological

Figure 2.1. (a) Typical spongiform change. H & E; (b) status spongiosus; (c) Spongiform vacuoles as seen by electron microscopy. Vacuoles contain curled membrane fragments and secondary chambers. Original magnification, ×12 000; (d) Intramyelin vacuole, Original magnification, ×12 000.



description of Jakob's cases based on studies of thick celloidin-embedded sections stained according to the Nissl technique revealed purely neurodegenerative process encompassing neuronal loss, central chromatolysis and astroglial proliferation with neuronophagia. Parenthetically, spongiform change were not visible by Nissl stain but re-appeared when the coverslips were removed and section re-stained with H & E.

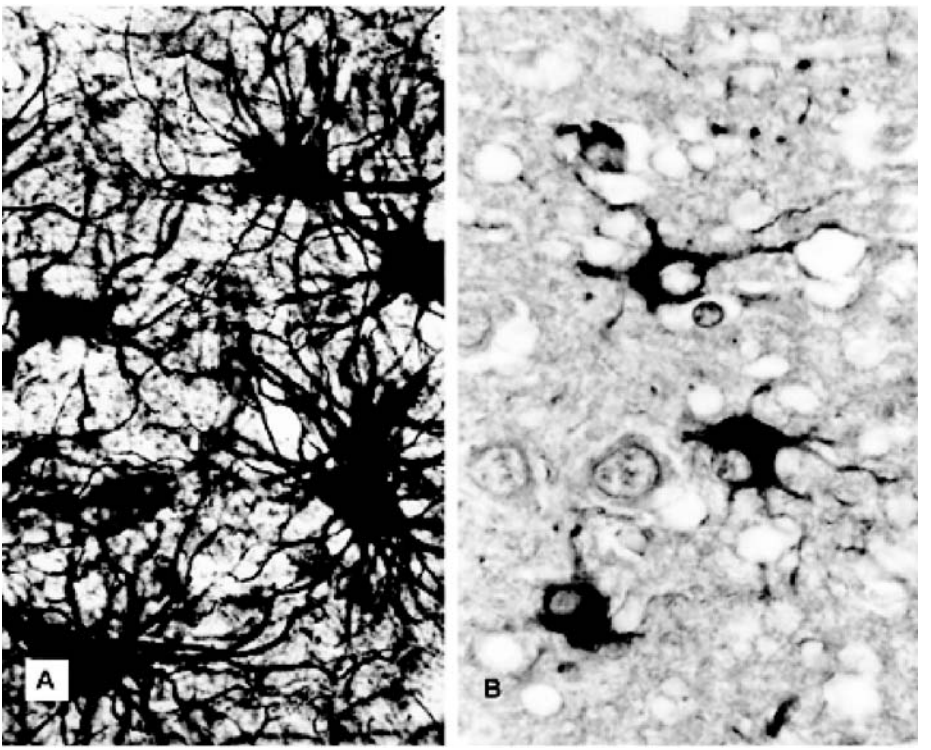
The classical triad of CHD neuropathology consists of vacuolation (spongiform change), neuronal degeneration (neuronal loss) and astrocytosis (Figure 2.1–2.2). The changes are bilaterally symmetrical but may be local and, occasionally, even unilateral[29].

2.6. Structural Changes

2.6.1. Spongiform changes

Most characteristic and even "semi-pathognomic" for CJD is the presence of spongiform change which remain well preserved even

Figure 2.2. (a) Dense astrocytic gliosis in a CJD case as revealed by Cajal gold sublimate method. Courtesy of Prof. Herbert Budka, Vienna, Austria; (b) GFAP-immunopositive astrocytes against a background of severe spongiform change. CJD brain biopsy.



in exhumed cases[30]. Spongiform change consists of small, round or oval vacuoles within neuropil (Figure 2.1); vacuoles are confluent and form typical "morula-like" aggregates. In the cerebral cortex, spongiform change is confined to the deep cortical layers; those vacuoles in the superficial cortical layers are characteristic for fronto-temporal lobar degenerations including Pick disease or are merely artefactual. It must be stressed, that in cases of longer duration spongiform change may be masked by the overall loss of neurons, collapse of the cortical cytoarchitecture and robust proliferation of astrocytes. To this end, Masters and Richardson[31] discriminated "spongiform change" from "spongiform state ("status spongiosus"),' the latter consisting of larger cavities of irregular shape in the neuropil (Figure 2.1b) between dense meshwork of proliferating astrocytes. Status spongiosus is not specific for TSEs and can occur in the end stage of a wide range of neurodegenerative disorders if widespread neuronal degeneration and loss has occurred.

In certain TSE, especially in fatal familial insomnia, vacuolation may be very limited and largely focal; in the latter example it is generally confined to some thalamic nuclei.

Ultrastructurally, vacuoles are always membrane-bound and contain secondary-vacuoles or "chambers" (vacuoles within vacuoles), "curled" membrane fragments and amorphous "fluffy" material of unknown composition (Figure 2.1c). The membranes lining the vacuoles may be simple or multiple[32]. Typical vacuoles originate in neuronal elements—mostly dendrites or, rarely, axons; those described in astrocytes seems to be fixation artifacts. Spongiform vacuoles have been also studies by scanning electron microscopy (SEM)[33] which revealed ulcerations and defective membranes as well as "rough elevated areas" corresponding to "amorphous membranes" as seen by TEM. SEM detected also small blisters that are equivalent to small vesicles by TEM.

The second type of vacuoles are those originated within the myelin sheath (Fig 1d)[34]. These are largely non-specific finding but they may be robust in the panencephalopathic type of CJD in which white matter is predominantly affected and its degeneration does not result from Wallerian degeneration. These intramyelin vacuoles are several times larger than diameter of average myelin fibre and looked "empty". Within distended myelin sheaths, shrunken axons are observed but many bullous swellings contained no axons. Some axons look normal but others were filled with neurofilaments and scanty electron-dense bodies. Still other axons are attached to the innermost myelin lamellae by a thin "neck" probably a mesaxon.

2.6.2. Astrocytosis

Variably severe astrocytosis is observed among almost all neurodegenerative conditions and CJD is no exception[35]. Hypertrophic astrocytes, detected by means of metal impregnation techniques (Holzer, Kanzler or Cajal's—Figure 2.2a) or more recently by immunostaining against glial fibrillary acidic protein (GFAP) (Figure 2.2b), are seen in all vacuolated areas. In cerebral cortex they are particularly prominent in deeper cortical layers, where swollen or gemistocytic forms are frequently observed. When destruction is so severe to lead to the collapse of vacuolated neuropil, proliferating astrocytes may virtually replace all other cellular elements. In such a situation the spongiform change may no longer be recognizable. In the cerebellum the proliferation of the astrocytes known as Bergman glia is frequently observed in a wide range of human TSEs.

Ultrastructurally, hypertophic astrocytes are no much different than those from other conditions. They are characterized by abundant glial

filaments within the cytoplasm. Liberski *et al.*[36] described close contacts between astrocytes and oligodendroglial cells; the pathophysiological significance of this phenomenon is uncertain.

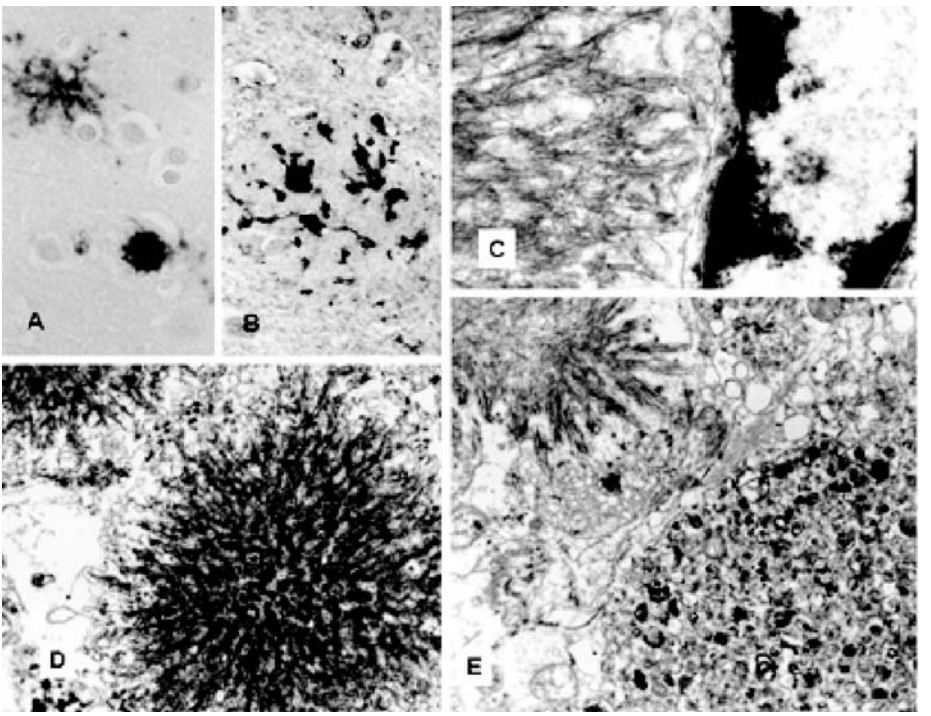
There are only two overlapping morphometric studies of astrogliosis in the cerebella (both Bergmann and velate astrocytes) in two cases of the ataxic form of CJD[37,38]. Astrocytes increased from 192.76 $\pm$ 117.98 per mm^2 in controls to 278.08 $\pm$ 137.73 per mm^2 in CJD. An increase in the cross sectioned nuclear area of Bergmann glia (32.72 $\pm$ 6.8 μm^2 vs 42.75 $\pm$ 9.61 μm^2) and of velate astrocytes (34.86 $\pm$ 7.29 μm^2 vs. 39.37 $\pm$ 7.10 μm^2) was seen when control values were compared with those of CJD values. Of note, the basic three-dimensional geometry of the astrocytic scaffold of the cerebellum was maintained despite severe loss of granule cells. Electron microscopy revealed several subcellular organelles, rare but otherwise typical for reactive astrocytes, single cilia consisting of ciliary shafts, clusters of interchromatin and perichromatin granules, various adhesive plaque junctions and simple and granular nuclear bodies. Of particular interest is the presence of infoldings of plasma membranes in the perivascular regions of astrocytic end-feet. These infoldings were covered by an interrupted or continuous electron-dense undercoat of 30–60 nm in diameter. The latter observation is in agreement with the earlier freeze-etching study of Dubois-Dalcq *et al.*[39] who showed an increased number of astrocyte-specific particles as opposed to their depletion on membranes forming vacuoles.

2.6.3. Amyloid plaques

In GSS, vCJD and in some murine scrapie models of disease, "classical" amyloid plaques are frequent (Figure 2.3), but are often absent in many human TSEs cases[40], in BSE and most ovine scrapie. The presence of amyloid (i.e., a protein in a β-sheeted conformation[12]) can be detected *in situ* by tinctorial stains for amyloids, including birefringence following staining with Congo red, immunohistochemistry for PrP, or, in brain homogenates, in the form of fibrillar PrP aggregates labelled *prion rods*[13]. However, on transmission electron microscopy most of the disease specific PrP^d identified in TSE affected brains by immunocytochemistry is not visibly fibrillar[41].

Typically, "classical" amyloid plaques consist of a congophilic PrP-immunopositive dense core of densely interwoven amyloid fibrils surrounded by different numbers of dystrophic neurites (DN). The amlyoid plaque usually exhibits positivity for other tinctorial stain including Periodic acid-Schiff (PAS), Alcian blue and various silver impregnation

Figure 2.3. (a) PrP^d -immunopositive amyloid plaque; (b) An amyloid plaque containing abundant microglial cells immunostained against ferritin. GSS case[42]; (c) an electron micrograph showing a microglial cell (its nucleus is visible in the upper part of the picture) in close contact with amyloid fibrils. Original magnification, ×30 000; (d) A typical stellate kuru plaques. Original magnification, ×12 000; (e) A plaque (upper right) from a GSS case[46]. In the lower left part a huge dystrophic neurite is visible. Original magnification, ×12 000.

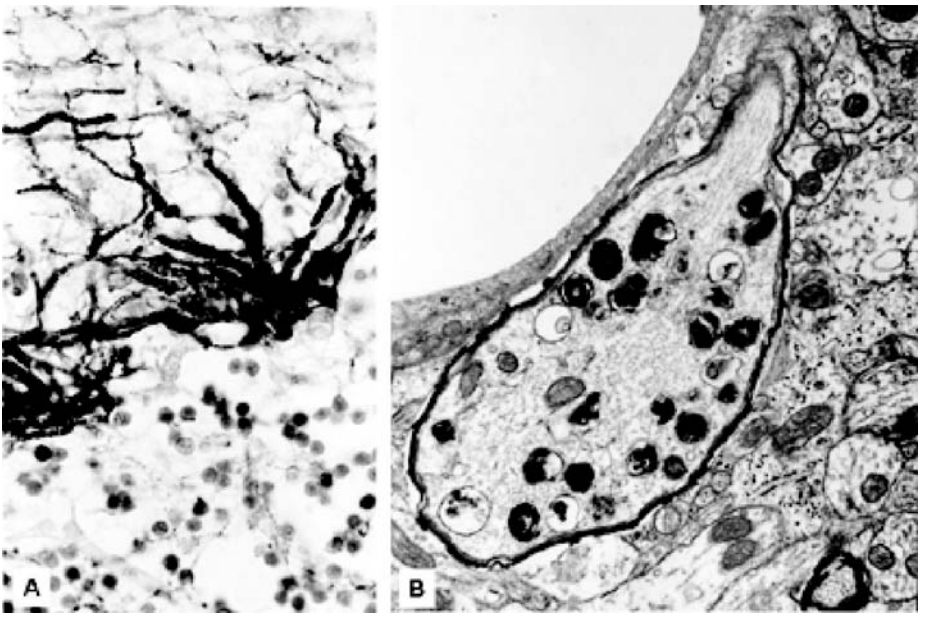


techniques. The PrP-plaque is penetrated by astrocytic processes. Microglial cells were detected in plaques of GSS[42] (Figure 2.3b-c) and in murine scrapie plaques[43]. The exact morphology of the PrP-plaque varies. Thus, the "kuru" plaque consists of stellate core with minimal numbers dystrophic neurites (or non at all) (Figure 2.3e)[44]. Multicentric plaques consisting of numerous such cores of different sizes and shapes *are typical of GSS*[45,46]. In vCJD, the large fibrillary amyloid plaques in the cerebral and cerebellar cortex are surrounded by a corona or "halo" of spongiform change, the so-called "florid" plaque[47].

2.6.4. Neuroaxonal dystrophy

Neuroaxonal dystrophy (NAD) is one form of neuronal degeneration[48] which may occur in apparently normal brains but became "pathological"

Figure 2.4. (a) a cluster of NFP-immunopositive neuritis in a case of CJD; (b) a dystrophic neurite containing numerous neurofilaments and immersed in them lysosomal electron-dense bodies. Original magnification, ×12 000.



only if found in increased numbers. The ultrastructural correlate of NAD is a dystrophic neurite—a neuronal process (a dendrite or a myelinated axon) filled with abnormal subcellular organelles—like lysosomal electron-dense bodies or masses of neurofilaments (Figure 2.4).

NAD was described in CJD[49], GSS[46] and in numerous experimental scrapie models. Dystrophic neurites in both natural and experimental CJD accumulate phosphorylated neurofilament proteins (Figure 2.4)[50].

2.6.5. Apoptosis and autophagy

As in many neurodegenerative diseases caused by the accumulation of "toxic" proteins such as Alzheimer's disease, neurons in TSEs die via programmed cell death of which only the apoptotic process is relatively well characterized. Three types of programmed cell death (PCD) can be discriminated[51].

1) First type (apoptosis), is characterized ultrastructurally by specific alterations—cell shrinkage, condensation of chromatin and, eventually, formation of so called "apoptotic bodies". The latter are actively phagocytosed by macrophages.

2) A second type is characterized by formation of numerous cytoplasmic autophagic vacuoles that are subsequently fused with lysosomes. The appearance of autophagy is followed by mitochondria dilatation and expansion of Golgi apparatus and endoplastic reticulum. The molecular mechanism is different that that of apoptosis and consists of complex interplay of numerous proteins including the Tor kinase.
3) A third type is similar to the second type, except for the negligible or absent involvement of lysosomes. Ultrastructurally, type 3 cell death is characterized by swelling of intracellular organelles resulting in the formation of large empty spaces within the cytoplasm. Of interest, TSEs are characterized of "spongiform vacuoles" formation within neuronal elements[52]. While the latter have never been linked to the type 3 PCD, the ultrastructural resemblance of "large empty spaces" to "spongiform vacuoles" appear to be worthy of studies.

Using TUNEL methodology, apoptotic neurons have been repeatedly shown in both naturally occurring and experimentally induced TSEs[53–55], and some investigators believe that at least a proportion of "dark neurons" may represent those cells undergoing apoptosis. The latter cells appear shrunken and of homogeneously dark cytoplasm. Their real significance is uncertain and many workers regarded them, however, as merely fixation artefacts. Furthermore, typical autophagic vacuoles have been reported by us[56], and by others[57,58] in CJD- and scrapie-affected rodent brains. Recently, autophagy was found in degenerating synapses in CJD[59].

Autophagy, being an evolutionarily ancient cellular response to intra- and extracellular noxious stimuli, may precede or co-exist with apoptosis, and the process may be induced by apoptotic stimuli. Furthermore, the level of autophagy may define the sensitivity of a given neuronal population to apoptotic stimuli, which may underlie the phenomenon of "selective neuronal vulnerability"[60]. Thus, autophagy and apoptosis are interconnected.

Cellular autophagy is a physiological degradative process employed, like apoptosis, in embryonic growth and development, cellular remodeling and the biogenesis of some subcellular organelles—viz. multilamellar bodies. Nascent immature autophagic vacuoles coalesce with lysosomes to form degraded autophagic vacuoles, and in apoptosis, only excessive or wrongly placed autophagy cause a pathological process. Of note, autophagy is highly enhanced in other brain amyloidoses, Alzheimer disease, Parkinson's disease, Huntington's disease, where the signal for autophagy is huntingtin. Here, we extend these observations using different model of scrapie and CJD[61].

2.6.6. Definition of autophagic vacuoles

Autophagic vacuoles are composed of areas of the cytoplasm sequestrated with single, double or multiple membrane (phagophores) originating from the endoplasmic reticulum. Sequestrated cytoplasm contains ribosomes, small secondary vacuoles, and occasional mitochondria. Some vacuoles presented a homogenously dense appearance.

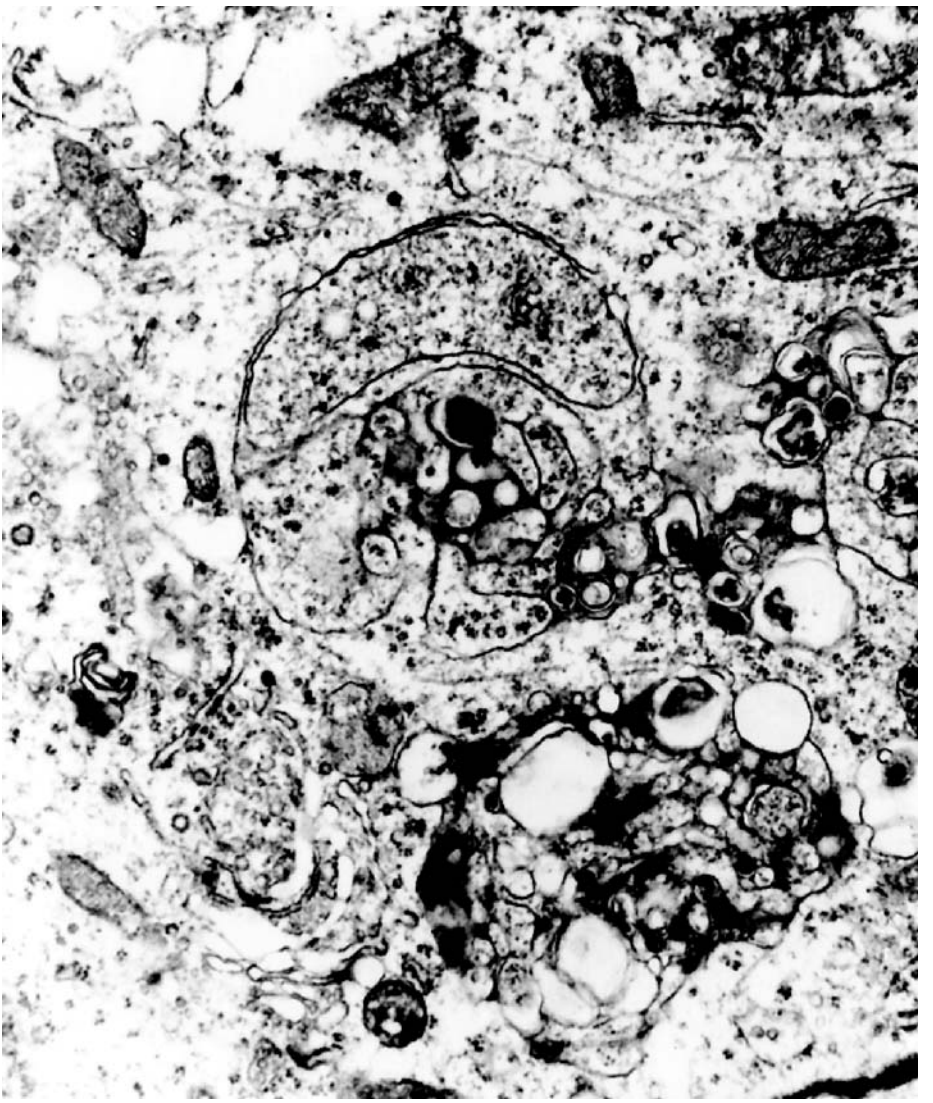
2.6.7. Formation of autophagic vacuoles in TSE experimental models

Both scrapie models used by us (the 263K and 22C-H) revealed similar frequencies and ultra-structural features of autophagic vacuoles, and will be described together. The various changes were observed simultaneously in different areas of the same sample but the following description is organised according to our interpretation of their chronological evolution. Initially, a part of the neuronal cytoplasm was sequestrated by concentric arrays of double membranes; the enclosed cytoplasm appeared relatively normal except that its density was often increased. In many affected neurons, electron density of the central area drastically increased. The membranes duplicated within the cytoplasm in a labyrinth-like manner and the area sequestrated by these membranes enlarged into a more complex structure consisting of vacuoles, electron-dense areas and areas of normally-looking cytoplasm connected by convoluted membranes (Figure 2.5). Of note, autophagic vacuoles form not only in neuronal perikarya but also in neurites and synapse[59]. We also observed, a large area of the cytoplasm transformed into a collection of autophagic vacuoles of different sizes and it seems that the latter may represent a final stage of autophagy as the number of such cells increased toward the terminal stage of the incubation period. In experimental CJD and GSS, autophagy was also seen albeit with much lower frequency.

2.6.8. Tubulovesicular structures

The *tubulovesicular structures* (TVS) (also known as the *scrapie-associated particles)* are the only structures unique at the level of thin-section electron microscopy for all TSEs so far examined and have been identified in GSS, CJD, many rodent scrapie models, BSE, and in natural scrapie of sheep[49,62–65]. TVS were first described in mice infected intracerebrally scrapie[66]. Of note, in natural scrapie in sheep TVS appeared as membrane-bound accumulations of round particles measuring 35 nm in diameter. The electron-dense core could be demonstrated in some of

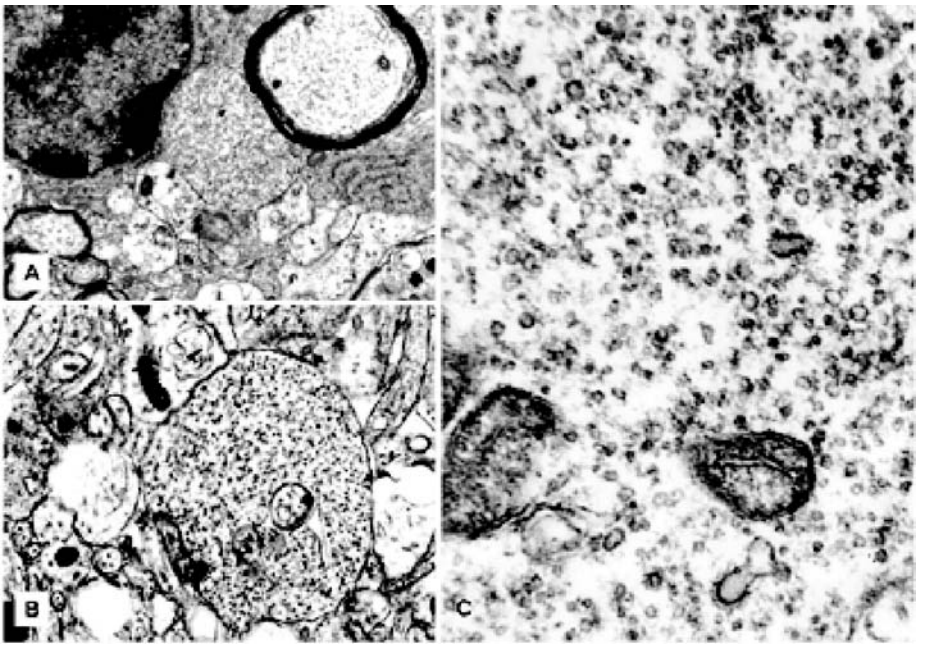
Figure 2.5. Typical autophagic vacuole consisting of convoluted membranes sequestrating a part of neuronal cytoplasm. Original magnification, ×12 000.



them[67] (Figure 2.2, inset). TVS have been reported in the majority of models of scrapie in rodents studied so far. In humans, TVS were found in CJD[49,68], GSS[63], vCJD and FFI[68].

At low magnification, a process containing TVS is crowded with structures typically of higher electron density than other elements in this field. In cross section, TVS are smaller than synaptic vesicles but larger than microtubules; their profiles are highly variable (Figure 2.6).

Figure 2.6. (a) Low power electron micrograph of distended terminal crowded with TVS. Original magnification, ×12 000; Lower (c) and higher (c) magnification of TVS. Note that TVS are highly pleomorphic. Original magnification, (a), ×12 000; (b), ×50 000.



The exact topology of TVS is not entirely clear. In most published electron micrographs TVS appeared as spheres measuring between 20 and 40 nm in diameter. Some investigators suggested that the tubular "arrays" of TVS result from overlapping spherical profiles, but we repeatedly shown short tubular forms of TVS, and it is therefore evident that TVS are pleomorphic structures existing in at least two forms—spheres and short tubules.

Only limited data are available concerning the number and density of TVS through the incubation time of experimental TSEs. In hamsters inoculated with the 263K strain of scrapie, TVS were initially observed as early as 3 weeks after i.c. inoculation, but their number increased only with the onset of disease at 9 to 10 weeks after inoculation[69]. Noteworthy, vacuolation and astrocytosis were detected at 8 weeks post-inoculation and, thus, followed the appearance of TVS.Similar findings were described by[64]. In mice infected with the Fujisaki strain of GSS, TVS were first seen 13 weeks after i.c. inoculation and their numbers increased dramatically at 18 weeks after inoculation, when the first signs of clinical disease were noted[69]. In contrast to 263K, the Fujisaki strain of CJD showed vacuolation and astrocytosis at the same time as the

appearance of TVS and the increase in the number of processes containing TVS paralleled the increasing intensity of vacuolation and astrocytosis. TVS were approximately twice as abundant in terminally ill mice following intraocular inoculation as in mice following intracerebral inoculation[69]. By contrast, final intensity of vacuolation and astrocytosis was not dependent on the route of inoculation. In conclusion, TVS appear early in the incubation period, preceding the onset of clinical disease. Furthermore, in scrapie-infected hamsters, TVS preceded the appearance of other neuropathological changes. The approximately 10^3-fold lower infectivity titre of the Fujisaki strain of CJD, compared to the 263K strain, may have accounted for the delayed appearance of TVS in experimental CJD. The apparent correlation between the number of neuronal processes containing TVS and infectivity titre may explain why in cell cultures infected with scrapie, TVS could not be found and why their number in experimental scrapie in sheep or CJD in humans was so low[49,65].

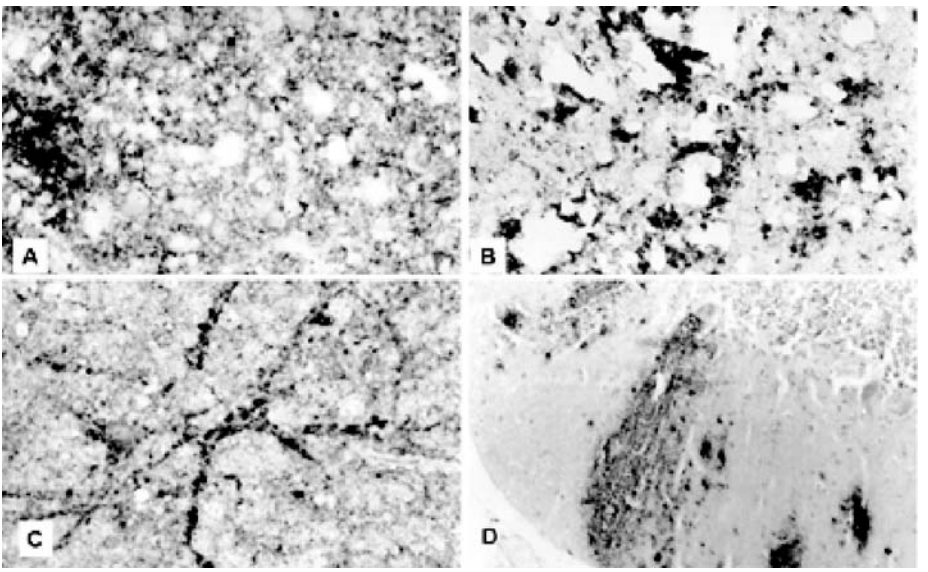
2.6.9. Biochemistry of TVS

The chemical composition of TVS is unknown. Earlier studies reported that ruthenium red enhances contrast of TVS. These staining properties of TVS may be interpreted as an evidence for the presence of glycosyl residues within TVS. To evaluate whether PrP is a part of TVS, we employed immunogold electronmicroscopy[70,71]. In all models TVS-containing processes were readily detected and neither these processes nor TVS themselves were decorated with gold particles. Even if amyloid plaques were observed in close contact with TVS-containing neuronal processes, the plaques were decorated with gold particles while the processes remained unstained[70,71]. At higher magnification, amyloid fibrils were clearly visible with numerous round or short tubular particles attached to them. At such a magnification, these particles were membrane-bound and their diameter was approximately twice that of amyloid fibrils; they were virtually indistinguishable from TVS. TVS located in areas adjacent to plaques in the 87V model and in areas of diffuse PrP immunolabelling in ME7 model were also unlabelled with anti PrP antisera.

2.7. PrP^d Immunohistochemistry

Immunohistochemistry (ICC) became the major diagnostic tool to detect PrP^{d21} along with its more refined equivalent the *histoblot*[72] and *paraffin-embedded blot* (PET) technique[73]. The major caveat of ICC

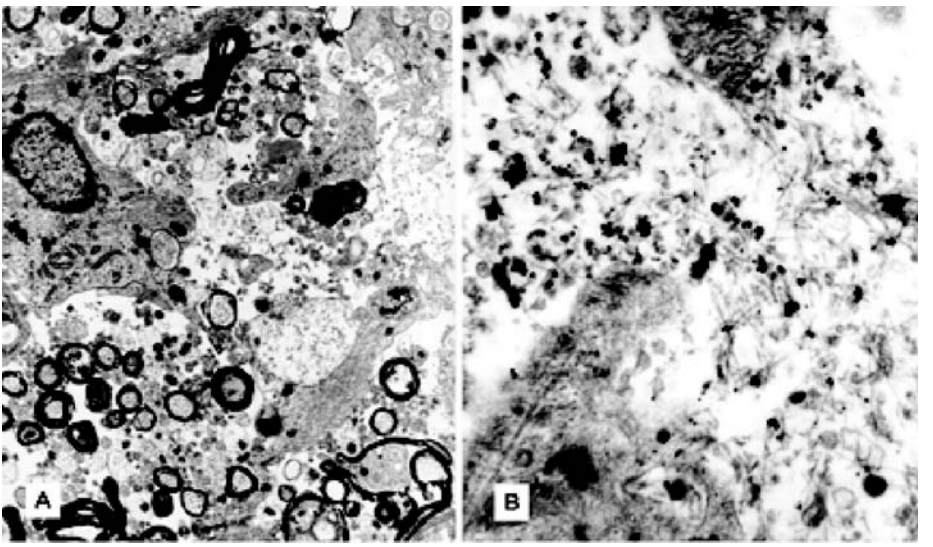
Figure 2.7. (a) Synaptic pattern of PrP^d accumulation (b) Perivacuolar PrP^d pattern of immunostaining; (c) Perineuronal PrP^d pattern of immunostaining. Note that large neuron in the middle has radiating processes decorated with PrP^d deposits; (20) Stripes of PrP^d immunoreactivity running perpendicularly to the surface of the cerebellum.



is to get rid of PrP^c as all available antibodies, including widely used and commercially available 3F4 and 6H8 antibodies, cannot discriminate between PrP^c and misfolded PrP^d. To this end, different unmasking techniques are used—hydrating or hydrolytic autoclaving, microwaving, formic acid or guanidinum thiocyanate incubation; a combination of several of preatreatments may give the best results.

Several patterns of PrP^d expression are revealed by ICC[21]. Those include: the most difficult to visualize, synaptic (Figure 2.7a), perivacuolar (Figure 2.7b), perineuronal (Figure 2.7c) and plaque-like. In certain familial forms of CJD, including the E200K mutation, stripes of PrP^d immunopositivity running perpendicularly to the surface of the cerebellar cortex is visible (Figure 2.7d)[74]. If plaques are visualized by a routine neuropathology (H & E, PAS- or Alcian blue stainings) these are labeled "plaques"; if they are detected only by ICC and are not visible by routine techniques, they are called "plaque-like". In chronic wasting disease[6] and in some experimental scrapie models[70,71], subependymal deposits (subependymal plaques) are visible. These correspond, ultrastructurally, to areas of low electron density containing haphazardly-oriented fibrils that, when stained with anti-PrP Abs, are heavily decorated with PrP-conjugated gold particles (Figure 2.8).

Figure 2.8. (a) A loose plaque in the subependymal location. Note amyloid fibrils in electron-lucent spaces; (b) a high magnification showing PrP^d-immunopositive amyloid fibrils floating in electron lucent spaces. Dark deposits in (b) correspond to anti-PrP-conjugated gold-silver enhanced particles[70]. Original magnification, (a), ×4400; (b), ×50 000.



In both models, perivacuolar PrP deposits are also visible. Recently, intraneuronal PrP^d deposits have been described[75–76]. The intraneuronal PrP^d deposits may exist as diffuse type in the neuronal perikaryon; large intracytoplasmic inclusion bodies reminiscent somehow Pick bodies, intracytoplasmic punctuate deposits and somato-synaptic dots. There is inverse relation between PrP^d accumulation in neurons and overall PrP^d-immunostaining; furthermore, cases with robust PrP^d-immunostaining and low intranuclear staining exhibit lower age of onset. Of note, similar intraneuronal PrP^d-immunostaining was observed in celiac, superior mesenteric and stellate ganglia of vCJD cases[77].

PrP^d is also present in the peripheral nervous system as well as in lymphoid tissue, including the spleen[78]. In CJD, PrP^d was detected in satellite cells and neurons of trigeminal ganglia[79], and in an adaxonal location (Schwan cells ?) in nerve fibers. Those deposits were scant and were observed in 1 of 9 CJD cases and in 1 GSS case[80]. The paucity of peripheral PrP^d deposition remains in strong contrast to the widespread expression of PrP^d in experimental scrapie both in sheep and in hamsters[81]. In contrast, using sensitive Western technique, PrP^d was readily detected in spleen in 10 of 28 CJD cases and in the spleen of

8 of 32 CJD cases[82]. Of note, peripheral accumulation of PrP^d seems to be more abundant in uncommon CJD variants—MM2—cortical or MV2 (see section on classification).

2.8. Particularities of disease

2.8.1. Kuru

Kuru[1], the first human neurodegenerative disease classified as a transmissible spongiform encephalopathy (TSE) was first reported to Western medicine in 1957 by Gajdusek and Zigas[83]. The first systematic examination of kuru neuropathology (12 cases) was published by Klatzo *et al.* in 1959[84]. Pathological changes were confined to the brain and spinal cord, with the cerebellum, pontine nuclei, thalamus, and spinal cord bearing most of the burden. Macroscopically, some brains were oedematous and leptomeninges were congested. Microscopically, neuronal changes observed in anterior motor neurons of the spinal cord, in different brain stem nuclei, in the cerebellum, and in the cerebral cortex were non-specific in nature but nonetheless sufficient for Klatzo *et al.* to draw a parallel between kuru and Creutzfeldt-Jakob disease.

Numerous neurons were either shrunken and hyperchromatic or, to the contrary, pale with dispersion of Nissl substance or intracytoplasmic vacuoles not unlike those seen in scrapie, BSE, and chronic wasting disease, but which are rather infrequent in human TSE. In the striatum, some neurons were vacuolated to such a degree that they looked “moth-eaten”. Neuronophagia was observed. A few binucleated neurons were visible and torpedo formation was noticed in the Purkinje cell layer, along with empty baskets that marked the presence of degenerated Purkinje cells[85]. In the medulla, neurons of the vestibular nuclei and the lateral cuneatus were frequently affected; the spinal nucleus of the trigeminal nerve and nuclei of VIth, VIIth, and motor nucleus of the VIth cranial nerves were affected less frequently while nuclei of the XIIth cranial nerve, the dorsal nucleus of Xth cranial nerve and nucleus ambiguous were relatively spared. In the cerebral cortex, the deeper layers were affected more than the superficial layers, neurons in the hippocampal formation were normal. In the cerebellum, the paleocerebellar structure (vermis and flocculo-nodular lobe) was most severely affected, and spinal cord pathology was most severe in the corticospinal and spinocerebellar tracts. Astro- and microglial proliferation was widespread; the latter formed rosettes and appeared as rod- or amoeboid types or as macrophages (gitter cells). Myelin degradation was observed in 10 of 12 cases. Interestingly, the significance

of vacuolar changes was not appreciated by Klatzo *et al.*[84], but "*small spongy spaces*", were noted in 7 of 13 cases studied by Beck and Daniel[85].

The most striking neuropathologic feature of kuru is the presence of numerous amyloid plaques, described as "*spherical bodies with a rim of radiating filaments''* and found in 6 of 12 cases studied by Klatzo *et al.*[84], and in "about three quarters" of the 13 cases of Beck and Daniel[85]; they became known as "kuru plaques". These measured 20–60 μm in diameter, were round or oval and consisted of a dark-stained core with delicate radiating periphery surrounded by a pale "halo". Kuru plaques were most numerous in the granular cell layer of the cerebellum, basal ganglia, thalamus, and cerebral cortex in that order of frequency. It is noteworthy that in Gerstmann-Sträussler-Scheinker disease (GSS) plaques are located in both the granular cell and molecular layers, whereas in CJD plaques are confined to granular cell layer. Kuru plaques are metachromatic and stain with PAS, Alcian blue, and Congo-red, and a proportion of them are weakly argentophilic when impregnated according to Belschowsky or von Braunmühl techniques. Klatzo *et al.*[84] reported that plaques were most readily visualized by Holmes' silver impregnation method. Of historical interest, another unique disease reported by Seitelberger[86] as *''A peculiar hereditary disease of the central nervous system in a family from lower Austria''* (germ. *Eigenartige familiar-hereditare Krankenheit des Zentralnervensystems in einer niederoosterreichen Sippe*) was mentioned by Neumann *et al.*[87] who was thus the first person to suggest a connection between kuru and GSS and kuru.

Recently, a renewed interest in kuru pathology has been provoked by the appearance of a variant form of CJD (vCJD) resulting from infection by the agent of bovine spongiform encephalopathy (BSE), that is also characterized by numerous plaques, including "florid" plaques—a kuru plaque surrounded by a corona of spongiform vacuoles. Hainfellner *et al.*[44] analyzed by means of modern immunohistochemistry the case of a young male kuru victim (Kupenota) from the South Fore region whose brain tissue had transmitted disease to chimpanzees, and McLean *et al.*[88] examined a series of 11 cases of kuru still in the archives of the University of Melbourne. In contrast to the classical studies described above, both papers stressed the presence of typical spongiform change present in deep layers (III–V) of the cingulate, occipital, enthorrinal and insular cortices, and in the subiculum. Spongiform change was also observed in the putamen and caudate, and some putaminal neurons contained intraneuronal vacuoles. Spongiform change was prominent in the molecular layer of the cerebellum, in

peraqueductal gray matter, basal pontis, central tegmental area, and inferior olivary nucleus. The spinal cord showed only minimal spongiform change.

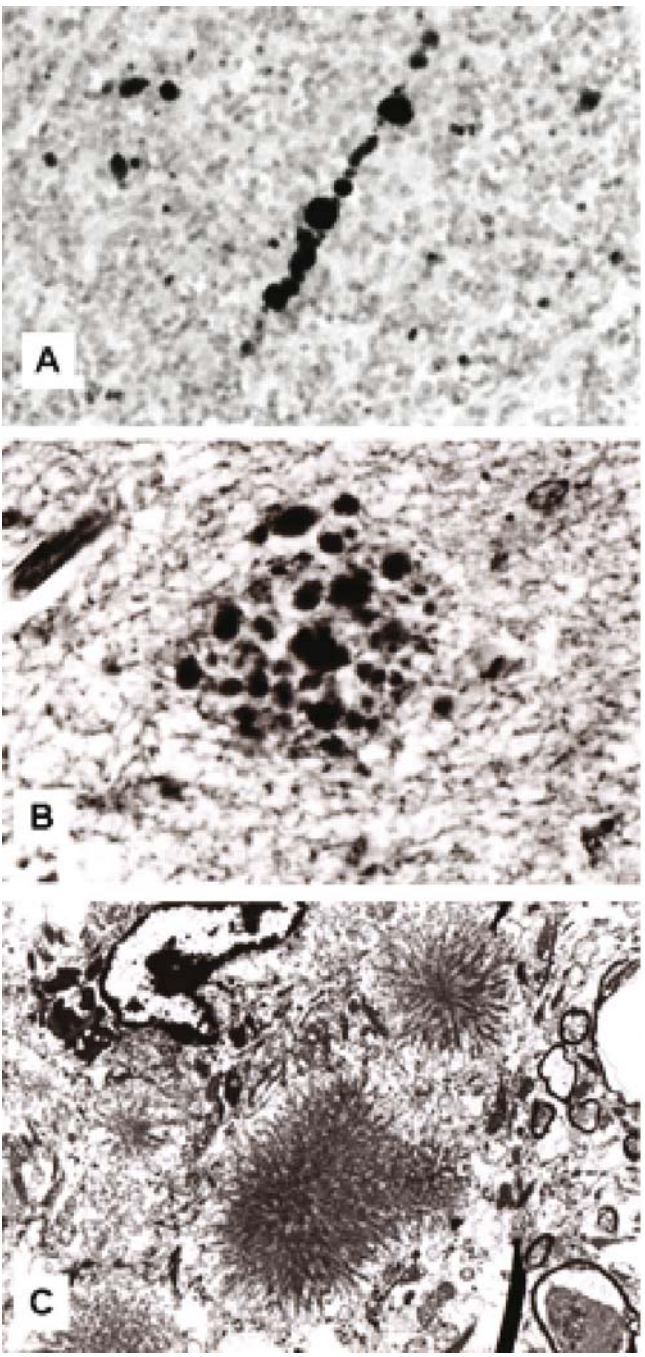
Immunohistochemical studies revealed that misfolded PrP was present not only in kuru plaques (Figure 2.9a), already demonstrated to be PrP^{Sc}-immunoreactive by Piccardo *et al.*[89], PrP^{Sc} but also in synaptic and perineuronal sites, and in the spinal cord the *substantia gelatinosa*was particularly affected, as in iatrogenic CJD cases following peripheral inoculation[90].

2.8.2. Gerstmann-Sträussler-Scheinker (GSS) disease

Gerstmann-Sträussler-Scheinker disease was described in 1936 and then it was re-evaluated by Seitelberger[86] and von Braunmühl[91] and transmitted to chimpanzees by Masters *et al.*[3]. In classical papers of Seitelberger[86] and Boellaard *et al.*[92] the similarities of GSS neuropathology to that of kuru was stressed and preconceived the transmission experiments of Masters *et al.*[3]. Following discovery of a mutation (Pro102Leu) in the *PRNP* gene by Hsiao *et al.*[93] many new mutations and new families have been described[94]. A detailed study of the first GSS family was published by Hainfellner *et al.*[45].

The detailed description of all diverse families is beyond the scope of this chapter and was reviewed elsewhere[94,95] and only general overview will be provided. The hallmark of GSS is multicentric plaque (Figure 2.9b–c). In contrast to CJD, where kuru plaques occur predominantly in the granular and the Purkinje cell layer, multicentric plaques are present in the molecular layer of the cerebellum[45,94,96]. As the name "multicentric" implies, GSS plaques are composed of several cores of different sizes merging together. In contrast to classical kuru plaques, multicentric plaques are neuritic, i.e., they are surrounded by dystrophic neurites immunolabeled against neurofilament proteins. In several families, dystrophic neurites contained also paired helical filaments (PHF)—identical to those found in dystrophic neurites in Alzheimer's disease[96,97]. At the level of electron microscopy, separate cores are readily discernible but overlapping. Multicentric plaques fulfill the criteria for amyloid—like kuru plaques they are congophilic and birefringent under polarized light. In addition, diffuse PrP^{d} deposits may be demonstrated by PrP-immunohistochemistry. PrP^{d} in diffuse (or amorphous) plaques is not yet fibrillised. Spongiform change is variable in GSS and its presence depends on the presence of 21 kDa PrP fragments. In classic description,

Figure 2.9. (a) A row of PrP^d-immunupositive kuru plaques[44]; (b) A typical multicentric plaque stained against PrP^d; (c) an electron micrograph of a cluster of several amyloid plaques from a GSS case[46], note a microglial cell in a vicinity; original magnification, $\times$4400.



GSS was regarded as "system degenerations"[86] and indeed, several white matter long tracts were found to degenerate in GSS.

2.8.3. Variant Creutzfeldt-Jakob disease

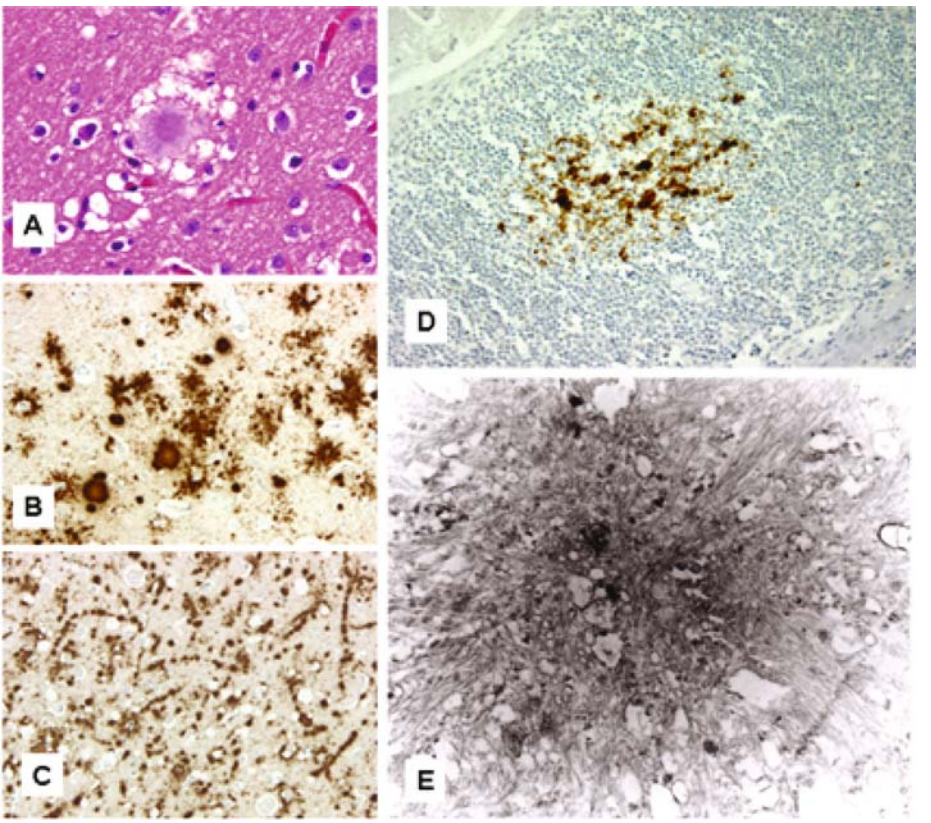
In 1996, Will *et al.*[47] reported a novel from of human prion disease which is now known as vCJD. Clinically, this disorder presents as a progressive neuropsychiatric syndrome with psychiatric and/or sensory symptoms at disease onset followed by ataxia, other movement disorders (particularly myoclonus), visual abnormalities and cognitive impairment, resulting in a terminal akinetic and mute state. Since its original description, 146 cases of vCJD have been identified in the UK, 6 cases in France and 1 in each of Canada, Ireland, Italy and the US. In the UK, the rate of new cases of vCJD has declined significantly over the past 12–18 months. Unlike most other forms of prion disease, this disorder predominantly affects young adults with an average age of onset around 28 years and a duration of illness of around 13 months. All patients who have been genotyped are methionine homozygotes at codon 129 in the prion protein gene.

The neuropathology of vCJD is distinct from other human prion diseases, and is characterized by multiple florid plaques. Florid plaques (Figure 2.10a) are fibrillary structure with a dense core surrounded by a pale region of radiating fibrils, and surrounded by a ring of spongiform change[47]. These plaques can be demonstrated using silver impregnation techniques and the PAS and Alcian blue stains[98]. Florid plaques occur in all layers of the cerebral cortex, but are most conspicuous at the bases of the gyri in the occipital and cerebellar cortex. They are also numerous in the molecular layer of the cerebellum.

Ultrastructural studies of the florid plaques in variant CJD have demonstrated the masses of radiating fibrils at the periphery, with abnormal neurites similar to those seen at the periphery of the Aβ plaques in Alzheimer's disease (Figure 2.10c)[99]. Neurofibrillary tangles and paired helical filaments have not been identified in variant CJD, and immunocytochemistry for tau gives negative results. Immunoelectron microscopy showed PrP accumulation both in the amyloid fibrils and in some of the abnormal cell membranes surrounding the plaques[100].

Spongiform change is widespread but often patchy within the cerebral cortex, mostly in a microvacuolar pattern or in relation to amyloid plaques. In contrast, confluent spongiform change is always present in the caudate nucleus and putamen, and focal spongiform change is present in most of the thalamic nuclei, the hypothalamus and globus pallidus, but the posterior thalamic nuclei (including the pulvinar) are

Figure 2.10. (a) A typical florid plaque from a case of vCJD, composed of a fibrillary amyloid core surrounded by small spongiform vacuoles; (b) PrP immunocytochemistry shows an intense positive reaction in florid plaques in vCJD. Many smaller plaques and amorphous PrP deposits are also demonstrated; (c) Perineuronal and linear periaxonal patterns of the PrP-accumulation in the basal ganglia in a vCJD case; (d) PrP-immunoreactivity in the tonsil of a vCJD case, showing staing of follicular dendritic cells within a germinal centre; (e) An electronmicrograph showing the florid plaque.



spared. Mild spongiform change is detected in the periaqueductal grey matter in the midbrain, in the pontine nuclei and in cerebellar cortex, often associated with amyloid plaques.

Neuronal loss in the cerebral cortex is most severe in the primary visual cortex. Neuronal loss in the basal ganglia is most evident in cases with severe and confluent spongiform change. In the thalamus, neuronal loss was most severe in the posterior nuclei, particularly in the pulvinar, which also showed marked astrocytosis[101]. The severe astrocytosis in the posterior thalamus was best visualized on immunocytochemistry for glial fibrillary acidic protein[101]. This technique also

demonstrated astrocytosis in relation to areas of severe neuronal loss and less frequently around the margins of amyloid plaques in other brain regions. Neuronal loss and astrocytosis were not conspicuous in the pons, medulla and spinal cord, but were variable in the cerebellum, sometimes most severe in the vermis.

2.8.4. Immunocytochemistry

The florid plaques in the cerebral and cerebellar cortex give an intense positive reaction on immunocytochemistry for PrP[98] (Figure 2.10b). Smaller "cluster plaques" are revealed by immunocytochemistry for PrP in all cases. PrP immunocytochemistry also shows a widespread amorphous pericellular deposition of PrP around small neurons in the cerebral and cerebellar cortex. In the basal ganglia there is a predominantly perineuronal and periaxonal pattern of PrP accumulation (Figure 2.10c). A synaptic pattern of immunoreactivity with occasional plaques is detected in the thalamus. In the brainstem and spinal cord, PrP positivity is present at all levels in the gray matter, particularly in the substantia gelatinosa. No PrP accumulation was detected in either the leptomeninges or the dura mater.

2.8.5. Quantitative neuropathology in variant CJD

Quantitative studies on the first cases of variant CJD confirmed the initial morphological descriptions and indicated that the measurable histological accumulation of abnormal PrP deposits in the cerebellum was far greater than in sporadic CJD cases[102]. Furthermore, the selective involvement of the posterior thalamus was demonstrated, with levels of astrocytosis far in excess of sporadic CJD cases[102]. Subsequent quantitative studies have demonstrated that in the cerebral cortex the spongiform change is consistently most pronounced in the occipital cortex, but the relationship between the spongiform change and the presence of PrP amyloid plaques varies in different brain regions[103,104]. Analysis of the spatial patterns of abnormal PrP deposition in variant CJD has found no significant differences between different regions of the cerebral cortex[105]. In addition to quantitative histology, there is the prospect of developing textural analysis techniques to investigate the differences in patterns of abnormal PrP deposition[106].

2.8.6. Non-CNS tissues

PrP accumulation is identified in the retina and optic nerve[107], spinal dorsal root ganglia and in the trigeminal ganglia, but peripheral sensory

Table 2.1. Pathological diagnostic features of variant CJD

1. Multiple florid plaques in H&E sections; numerous small cluster plaques in PrP stained sections.
2. Amorphous pericellular and perivascular PrP accumulation in the cerebral and cerebellar cortex.
3. Severe spongiform change; perineuronal and axonal PrP accumulation in the caudate nucleus and putamen.
4. Marked astrocytosis and neuronal loss in the posterior thalamic nuclei and midbrain.
5. Reticular and perineuronal PrP accumulation in the grey matter of the brainstem and spinal cord.
6. Predominance of di-glycosylated PrP^{RES} in central nervous system and lymphoid tissues.
7. PrP^{RES} accumulation in germinal centres within lymphoid tissues throughout the body.

and motor nerves contain no detectable PrP. Immunocytochemistry for PrP in other organs (adrenal gland, thyroid gland, parathyroid gland, skeletal muscle, bladder, testes, pelvic organs (vagina, cervix, uterus, Fallopian tubes and ovaries), heart, lung, liver, kidney, oesophagus, stomach, pancreas, gall bladder, salivary gland and skin is negative[98,108].

In contrast, PrP accumulation is identified in follicular dendritic cells and macrophages within many germinal centres in the tonsils (Figure 2.10d) and within germinal centres in the appendix, Peyer's patches in the ileum, spleen and lymph nodes from the cervical, mediastinal, para-aortic and mesenteric regions and the thymus[98,108,109].

Neuropathological studies were important in identifying variant CJD as a novel human prion disease[47]. The diagnostic pathological features of variant CJD are summarized in Table 2.1.

Although florid PrP amyloid plaques have been described in cases of iatrogenic CJD following dura mater graft procedures[110], their number and distribution in the brain is more restricted than in variant CJD. In addition, the biochemical features of abnormal PrP in the brain in variant CJD on Western blot examination is relatively uniform[108] (in contrast to sporadic CJD, where multiple PrP isotypes have been identified[111,112]). These findings reinforce the need for detailed characterisation of human prion diseases by clinical, pathological and biochemical studies, which can be reinforced by experimental transmission to demonstrate infectivity and to undertake strain typing studies. This has been reinforced by the identification of a recent case of vCJD in a recipient of a blood transfusion from a donor who has died earlier (after donation) from vCJD[113]. Strain typing studies on the first cases of variant CJD allowed the early recognition of the similarities in the transmissible agent in BSE and variant CJD.

These findings have been supported by other independent workers, reinforcing the link between these disorders[14,114,115,116]. As BSE has now been identified in many other countries across the world, it is possible that more cases of vCJD will be identified outside the UK. The future for vCJD in the UK remains uncertain, since it is not clear if the other codon 129 subgroups will be susceptible to this disease. If so, increased numbers of cases might occur over an unknown time period, indicating a need for continued surveillance for CJD, at least in the near future.

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