Cell death and autophagy in prion diseases (transmissible spongiform encephalopathies)

Pawel P. Liberski¹, David R. Brown¹, Beata Sikorska¹, Byron Caughey¹, Paul Brown⁴
¹Department of Molecular Pathology and Neuropathology, Chair of Oncology, Medical University of Lodz, Poland; ²Department of Biology and Biochemistry, University of Bath, Bath, UK; ³Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute for Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA; ⁴retired, USA


Abstract

Neuronal autophagy, like apoptosis, is one of the mechanisms of programmed cell death. In this review, we summarize current information about autophagy in naturally occurring and experimentally induced scrapie, Creutzfeldt-Jakob disease and Gerstmann-Sträussler-Scheinker syndrome against the broad background of neural degenerations in transmissible spongiform encephalopathies (TSEs). Typically a sequence of events is observed: from a part of the neuronal cytoplasm sequestrated by concentric arrays of double membranes (phagophores); through the enclosure of the cytoplasm and membrane proliferation; to a final transformation of the large area of the cytoplasm into a collection of autophagic vacuoles of different sizes. These autophagic vacuoles form not only in neuronal perikarya but also in neurites and synapses. On the basis of ultrastructural studies, we suggest that autophagy may play a major role in transmissible spongiform encephalopathies and may even participate in the formation of spongiform change.

Keywords: autophagy, apoptosis, prion diseases, neurons, ultrastructure.

Introduction

The transmissible spongiform encephalopathies (TSEs), or prion diseases, are a group of neurodegenerative disorders which include kuru (Fig. 1) [73,89,135], Creutzfeldt-Jakob disease (CJD) [76], Gerstmann-Sträussler-Scheinker (GSS) disease [157], and fatal familial insomnia (FFI) in humans [147], natural scrapie in sheep (Fig. 2), goats [53,54,57], and mouflons [224], transmissible mink encephalopathy (TME) in ranch-reared mink [35], chronic wasting disease (CWD) of mule deer and elk in North America [137,221,222], bovine spongiform encephalopathy (BSE) or “mad cow disease” [218], and its analogues in several exotic species of antelopes [55,68,106,115] and wild felids in zoological gardens [223], and feline spongiform encephalopathy (FSE) in domestic cats [226].

The cause of these disorders is still not completely understood. Despite a wide acceptance for the prion
theory, the name of the infectious agent (a “prion”) still reflects our ignorance or at the very least, our uncertainties of its nature [44,139,152]. Those who prefer to view this pathogen as composed predominantly or exclusively of a pathologically misfolded protein use the term “prion” [184]; hence the term “prion diseases” [67,217]. In this review, we will use the term “PrP$^\text{Sc}$” to designate the normal soluble and proteinase K-sensitive cellular protein, and the term “PrP^\text{res}” to designate all of its pathological forms, including intermediate species that are sensitive to proteinase K (PK) digestion as well as the mature amyloid species that is insoluble and partially PK-resistant [32].

Other hypotheses, albeit less widely accepted, are still not formally rejected. The “virino” hypothesis suggests that the pathogen is a molecular chimera composed of a yet-to-be-discovered nucleic acid and a shell-protein, which is host-encoded (could be PrP) [113]. The fact that RNA enhances conversion of PrP into PrP$^\text{res}$ in vitro is interesting in this context [56]. The “unified theory” of Weissmann [216], not unlike the virino theory, suggests that the agent is a molecular chimera in which PrP$^\text{res}$ confers infectivity and an unidentified oligonucleotide specifies strain characteristics. The virus hypothesis simply suggests that the pathogen is a yet-to-be-identified unconventional virus [58,72,216]. To this end, virus-like particles have been repeatedly shown in all TSEs studied so far [140,144,153].

PrP, the PrP gene, the “prion hypothesis” and strains of the pathogen

PrP$^\text{c}$ is a highly conserved sialoglycoprotein encoded by a cellular gene mapped to chromosome 20 in man (Prion Protein; PRNP) and chromosome 2 in mouse (Fig. 3) [7,16,45,90,176,202,204]. The gene is ubiquitous [193,194,225]; it has been cloned from numerous mammalian species, included marsupials, and there are analogues of this gene in birds [71,93], reptiles [200], amphibians [209], and even fish [177]. The molecular biology of PrP has been extensively studied. Human PrP$^\text{c}$ contains 253 amino acids encoded by an intronless open reading frame (ORF) (Fig. 3) [7]. As a result, alternative splicing does not occur. Three forms of PrP$^\text{c}$ exist – one completely translocated and two transmembrane variants, CtmPrP and NtmPrP [94,95,214] (Fig. 4) – and the sequence encoding residues 151-165 that form the transmembrane region is highly conserved [214]. Furthermore, PrP undergoes endoproteolytic cleavage to yield 17 kDa N-terminally truncated form C1, while PrP$^\text{res}$ yields a slightly larger peptide designated C2 (Fig. 3) [43].

The “prion” hypothesis, which is deeply rooted in the association between PrP$^\text{res}$ and infectivity, was formulated by Stanley B. Prusiner in 1982 [10,59,161,181,182]. 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 was presented a decade earlier by Gibbons and Hunter [75], Griffith [85], and Levine [130], who all expanded the earlier suggestion of Alper and her co-workers [1], based on irradiation studies, that scrapie agent was devoid of disease-specific nucleic acid. Furthermore, other investigators in the late 1970s and early 1980s had found that scrapie infectivity was sensitive to proteolytic digestion [47,166].
Neuronal cell death in prion diseases

**Fig. 3.** Scheme of PrP generation. The *PRNP* gene (the name *PRP* was already used for Prolyine Rich Protein) is located on chromosome 20. There are 2 exons in humans (in some other species such as mice and cattle, three) and the whole open reading frame (ORF) is located within exon 2 (or 3 in 3-exon species). During the maturation process two glycans (CHO) at positions 181 and 197 are added. The final product is a cellular isoform of PrP (PrP<sup>c</sup>) which is converted into pathological isoform (PrP<sup>TSE</sup>). PrP<sup>c</sup> undergoes endoproteolytic cleavage (red arrows) to yield 17 kDa N-terminally truncated form C1 while PrP<sup>TSE</sup> yields a slightly larger peptide designated C2. Following proteinase treatment (black arrows), PrP<sup>c</sup> is completely degraded whereas PrP<sup>TSE</sup> is only partially degraded and yields a “core protein” designated PrP 27-30.

The PrP species found to copurify with scrapie infectivity, PrP 27-30, is a proteolytic cleavage product of pathologically misfolded protein, PrP<sup>TSE</sup>. However, PrP<sup>TSE</sup> is *not* the *primary* product of the cellular gene, which encodes both PrP<sup>c</sup> and PrP<sup>TSE</sup> (Fig. 3). The amino acid sequences and posttranslational modifications, i.e. glycosylation and a glycoposphatidylinositol (GPI) anchor, of PrP<sup>c</sup> and PrP<sup>TSE</sup> are identical, but their physico-chemical characteristics are strikingly different [207,208]. In particular, PrP<sup>c</sup> is completely degraded by limited proteolysis, but PrP<sup>TSE</sup> is only partially degraded, yielding a core protein (PrP 27-30), which may be visualised by electron microscopy as amyloid-like fibrillar structures called scrapie-associated fibrils (SAF) (Fig. 5) [163,164], currently better known as prion rods [185].

**Fig. 4.** Three different forms of PrP. Completely translocated and two transmembrane variants – *comPrP* and *memPrP*. C – C-terminal, N – N-terminal, M – membrane.
Cellular trafficking of PrP$^c$ and PrP$^{TSE}$

PrP$^c$, as is true of all cell surface glycoproteins, is synthesized first in the endoplasmic reticulum, matured in the Golgi apparatus, and transported in membrane-bound vesicles to the plasma membrane, where it is usually anchored by the GPI moiety (Fig. 6). Once it is on the cell surface, PrP$^c$ can be subjected to endocytosis and either degraded in lysosomes or recycled to the plasma membrane via recycling endosomes [92,149,150,180,198]. In peripheral (PNS) and central nervous system (CNS) neurons fast anterograde and retrograde axonal transport of PrP$^c$ have also been detected [11,170,171], facilitating movement to and from neural extremities.

Coincident with becoming infected, cells can bind and internalize exogenous PrP$^{TSE}$ aggregates [97,98]. Depending on cell type, PrP$^{TSE}$ internalization can involve heparan sulphate [97,98], laminin receptor [169], and/or ferritin transporters [167]. However, the presence of PrP$^c$ does not seem to be required as a receptor for exogenous PrP$^{TSE}$ [97,149].

![Scrapie-associated fibrils](image)

**Fig. 5.** Scrapie-associated fibrils (lower arrow) visualized by negative-staining electron microscopy. An upper arrow points to protofilaments. Original magn. × 30 000; bar = 100 nm
Once cells are infected with a TSE agent in vitro, new PrP\textsuperscript{Sc} formation appears to occur on the plasma membrane and/or along an endocytic pathway to lysosomes [12,13,41,42]. However, in cells expressing familial TSE-associated PrP\textsuperscript{c} mutants, the acquisition of PrP\textsuperscript{Sc}-like properties can begin to occur spontaneously at the earliest stages of PrP biosynthesis [92]. In TSE-infected animals, it appears that PrP\textsuperscript{Sc} can also be formed at extracellular sites. This is especially likely in scrapie-infected transgenic mice that express only anchorless PrP\textsuperscript{c} that is secreted from cells rather than bound to membranes by the GPI moiety [46]. In these mice, PrP\textsuperscript{Sc} accumulates almost exclusively in large extracellular amyloid plaques, which are the likely sites of conversion to PrP\textsuperscript{Sc}.

Because the spread of TSE infections and PrP\textsuperscript{Sc} from peripheral sites to the central nervous system follows neuroanatomical pathways [114,159, for review, see ref. 81], it is important to understand the neuronal transport mechanisms. Axonal transport processes, but not the fast form, appear to be involved [5,6,118,125,186]. However, the precise mechanisms by which PrP\textsuperscript{Sc} is transported between and within neural cells have not been well defined in vivo. Recent studies in cultured neuronal cells have shown that endocytosed exogenous PrP\textsuperscript{Sc} can be found in Rab7-positive late endosomes and transported in acidic vesicles along neurites to points of contact with other cells [149]. Other in vitro studies have provided evidence that PrP\textsuperscript{Sc} can be released from cells [195] in infectious particles that are vesicular and exosome-like (Fig. 7) [3,64,195]. Membrane-associated scrapie infectivity is considerably more efficient than purified PrP\textsuperscript{Sc} at inducing persistent PrP\textsuperscript{Sc} production in cultured cells [3]. Small membranous particles such as exosomes (Fig. 6) can be released from multivesicular exosomes.

**Fig. 6.** Cellular trafficking of PrP. PrP is synthesized first in the endoplasmic reticulum (ER), matured in the Golgi (cis, medial and trans; red arrow) apparatus, and transported in membrane-bound vesicles to the plasma membrane, where it is anchored by the GPI moiety. Once on the cell surface, PrP can be subjected to endocytosis and either degraded in lysosomes or recycled to the plasma membrane via recycling endosomes. PrP may also be transported through the membrane (yellow arrow) as multivesicular-bodies-derived exosomes. In TSEs, PrP\textsuperscript{Sc} accumulates extracellularly (green arrows) as plaques (A) or different forms of deposits (B). A plaque in “A” is a florid plaque of vCJD. Deposits in “B” are perineuronal deposits from Echigo-1 strain of CJD passaged in Syrian hamsters.
Fig. 7. (A) Synaptic pattern of PrP<sup>sc</sup> deposition and (B) plaques in kuru affected brain. Arrows in “A” point to focal accumulations of PrP<sup>sc</sup>. Plaques in “B” are from a kuru specimen kindly provided by D. Carleton Gajdusek. Bars, 0.5 μm
bodies and fuse with the membranes of other cells, providing a potential mechanism for the spread of infectivity between cells [3,4,64].

Deposition of pathologically misfolded protein

Deposits of misfolded PrP\textsuperscript{TSE} that accumulate within the central (CNS) and peripheral nervous system (PNS) and lymphatic tissues [138] correlate with infectivity in most but not all situations [127,154]. Immunohistochemistry (IHC) has become the major diagnostic tool for human prion diseases, by allowing the detection of PrP\textsuperscript{TSE} in fixed tissue sections [33]. More refined approaches, i.e. the histoblot [211] and paraffin-embedded blot (PET) techniques, can also be helpful [196]. A major hurdle with IHC is the elimination PrP\textsuperscript{c} labelling as no available antibodies, including the widely used and commercially available antibodies, can discriminate between PrP\textsuperscript{c} and misfolded PrP\textsuperscript{TSE} in tissue sections.

Several patterns of PrP\textsuperscript{TSE} expression are revealed by IHC including synaptic (the most difficult to visualize; Fig. 7a), perivascular, perineuronal (Fig. 7) and plaque-like [33]. If amyloid is visualized by routine neuropathology (H & E, Congo red, PAS- or Alcian blue staining), it is designated as “plaques” (Fig. 6, 7b); if detected only by IHC and not visible by routine techniques, it is called “plaque-like deposits”.

Neuronal cell death in TSEs

The premature, primary death of nerve cells underlies the clinical symptoms of prion diseases. Unfortunately, despite the great efforts of researchers, the cellular pathways leading to this neuronal loss are not entirely clear. What is more, the question whether there is a direct relation between the deposits of PrP\textsuperscript{TSE} and the loss of neurons still remains conjectural. As in other neurodegenerative diseases, in TSEs apoptosis has become the most popular concept of cell death. However, there is no direct and convincing evidence of apoptosis of nerve cells in most of the neurodegenerative diseases. In addition, the term “apoptosis” is used in a wider sense than it was originally coined and it has become synonymous with non-necrotic cell death or even with programmed cell death. The data on the role of apoptosis in prion diseases are conflicting. Among recently recognized other types of programmed cell death only autophagy has been reported in TSEs but its role in prion diseases pathology is not established.

There is currently no consensus on the classification of different types of programmed cell death. One of the oldest but also still considered most accurate classifications is based on morphology. According to this classification introduced by Schweichel and Merker [197], three types of programmed cell death (PCD) are discriminated [116,189,215,230]:

1) apoptosis,
2) autophagy,
3) cytoplasmic cell death.

Although it must be stressed that this categorization was based on the ultrastructural features of embryonic cells during morphogenesis, such a classification is widely accepted for developing and mature organisms.

1. The term “apoptosis” in its original meaning refers to a morphological phenomenon [111] characterized by chromatin condensation, cell shrinkage, pyknosis, plasma membrane blebbing and fragmentation of the nucleus (karyorrhexis). There is little or no ultrastructural modification of other subcellular organelles. The integrity of plasma membrane is maintained until the late stages of the process [124]. In the end-stage, the cell breaks into small membrane-bound fragments, called apoptotic bodies, which are phagocytosed by macrophages or, in the case of neurons, by microglial cells without inciting any inflammatory response [111].

2. Autophagic cell death is also one of the programmed cell death mechanisms, sometimes called “type II programmed cell death”, in contrast to “type I programmed cell death” (apoptosis). Contrary to apoptosis, autophagic cell death is characterized by abundant autophagic vacuoles in the cytoplasm, mitochondrial dilatation, and enlargement of both the Golgi apparatus and endoplasmatic reticulum, which precedes nuclear destruction. Intermediate filaments and microfilaments are largely preserved [36,37,99].

3. A third type of programmed cell death is called “cytoplasmic cell death” and it was subsequently
divided into two subtypes, 3A and 3B [49]. Type 3A of neuronal death that occurs during development is characterized by swelling of subcellular organelles, formation of large empty spaces within the cytoplasm, and fusion of these spaces to extracellular space and, finally, disintegration of the cellular structures. There are no features of autophagic or heterophagic activity. In the 3B subtype of cell death there are similar vacuoles and empty spaces in the cytoplasm but, in addition, there is a retraction of plasma membrane and karyolysis.

According to a recent review [162], it is now possible to discriminate eleven pathways of cell death occurring in mammals. These types of cell death include: 1) necrosis, 2) apoptosis, 3) anoikis, 4) caspase-independent apoptosis, 5) autophagy, 6) Wallerian degeneration, 7) excitotoxicity, 8) erythropoiesis, 9) platelet cell death, 10) cornification and 11) lens cell death. All of them, except for necrosis, are genetically programmed; however, morphological features of necrosis are occasionally observed during the active cell processes [158]. Some of those subtypes occur merely in one type of cell but others are more common. The first seven types are observed in nerve cells. The latter classification is also based on morphological features because in the majority of cases the molecular mechanism is not known [162]. Other investigators have discriminated more types of cell death, e.g. paraptosis, pyroptosis, Oncosis, abortosis, aposklesis and many more [66,168,188,205,228]. Recently, the Nomenclature Committee on Cell Death has been established and according to its suggestions the whimsical names of cell death should be replaced by more descriptive terms [123].

**Prion proteins and the cell death**

Mechanistically, neuronal cell death in prion disease has a remarkable feature that distinguishes it from neuronal loss in other neurodegenerative disorders. In the absence of cellular expression of PrP, neuronal death does not occur [103]. This was initially shown in cell culture systems with neurons derived from PrP-knockout mice [23,78], and later confirmed using an animal model in which PrP<sup>WT</sup> was transplanted into PrP-deficient tissue without evidence of neuronal death [15]. Even more complicated genetically engineered models have demonstrated that when cellular expression of PrP<sup>+</sup> is switched off, cell loss does not occur, and disease progress is abated; illness is entirely prevented when neurones cannot produce PrP<sup>+</sup>, even when non-neuronal tissue contains dense deposits of PrP<sup>WT</sup>. Neuronal protection against cell death can also occur in an animal model in which a different form of PrP<sup>+</sup> is expressed that is more difficult to convert to PrP<sup>WT</sup> because of a species barrier. In transgenic mice co-expressing hamster and mouse PrP<sup>+</sup>, infection with hamster prion results in production of PrP<sup>WT</sup> without any pathology or neuronal death [187]. This suggests that expression of a form of PrP<sup>+</sup> resistant to protein conversion protects cells from neurotoxicity. However, if the transgenic mice only express hamster PrP<sup>+</sup> in astrocytes, then infection with hamster prions results in prion diseases with associated neuronal death. In this case, the neuronal death is possibly indirect as a result of increased neuronal sensitivity to glutamate toxicity [18].

The potential for PrP<sup>+</sup> expression to protect against neuronal cell death runs counter to the finding that expression is necessary for susceptibility to neuronal death in prion disease. The only logical explanation is that its normal cellular function is protective. Loss of that function due to loss of expression of the protein would result in alterations in the expression of proteins with a similar function, resulting in a compensatory effect. However, loss of function of the protein with continued expression, as occurs in prion disease, does not result in an increase in the compensatory mechanisms. It has been postulated that PrP<sup>+</sup> is an antioxidant (increased expression of other antioxidants has been noted in PrP-knockout mice) [26,219]. Simple loss of function is clearly not the cause of cell death in prion disease, as PrP-knockout mice do not show altered cell survival in vivo [34]; however, neurons in culture are more susceptible to a range of neurotoxic insults when they are derived from PrP-deficient mice [24,26,27,126,219].

**Aggresomes**

It is well known that protein aggregates are generally difficult to unfold or to degrade. Misfolded and aggregated proteins are usually handled in the cell through chaperone-mediated refolding or, when this is impossible, they are destroyed by proteasomal degradation. Recent findings suggest that there is...
a third way for a cell to deal with misfolded proteins. This pathway involves the sequestration of aggregated proteins into specialized “holding stations”, as they are sometimes called, or aggresomes. In this mechanism, proteins form small aggregates that are transported along microtubules (MTs) towards a microtubule organizing centre (MTOC) by a process mediated by the minus-end motor protein dynein. At the organizing centre, the particles form a spherical structure, usually 1-3 μm in diameter, called an aggresome. Aggresomes are not just static garbage deposits; they recruit various chaperones, ubiquitination enzymes, and proteasome components. They are also supposed to trigger autophagy [74].

A recent report of Kristiansen et al. [122] suggests that neuronal propagation of prions invokes a neurotoxic mechanism involving intracellular formation of PrP\textsuperscript{\textalpha} aggresomes. The authors showed that only in prion-infected cells did mild proteasome impairment result in formation of large cytosolic, perinuclear structures, containing PrP\textsuperscript{\textalpha}, heat shock protein 70, ubiquitin, proteasome subunits and vimentin. These structures are consistent with the definition of aggresomes. Those authors also claimed to show aggresomes in vivo in brains of prion-infected mice, but it is well known that vimentin is present in neurons only in trace quantities while it is robust in glial cells. This means that showing aggresomes in vivo needs further studies. A few years earlier Cohen and Taraboulos [50] showed that hampering the activity of cyclophilin isomerases with the fungal immunosuppressant CsA in different cell lines led to accumulation of a PrP population with prion-like properties that was not ubiquitylated and partially resisted proteasomal degradation. These aggregated molecules formed perinuclear aggresomes. Although a growing body of evidence seems to confirm the formation of aggresomes in prion diseases, it must be mentioned that the majority of the studies were performed in vitro and PrP\textsuperscript{\textalpha} in human or animal diseased brains does not intracellular aggregates reminiscent of aggresomes. Similar aggregates are only observed by electron microscopy, but they are extremely rare.

**PrP and Oxidative Stress**

Recent work on cell death mechanisms has focused on pathways involving PrP\textsuperscript{\textalpha}. Numerous cell culture lines exposed to neurotoxic PrP fragments have not always given consistent results, although it appears that caspases are involved [96,122,191,201], and that levels of ERK proteins are increased when cells are treated with toxic forms of PrP [129]. Other proteins have also been suggested to play a role, such as p38 [52], JNK, and Bax. However, none of these observations have been independently confirmed. Several of the studies have also suggested that calcium entry is involved [24,172,175,213], but this is a common event of many cell death pathways and the true intracellular pathway has yet to be determined.

It seems likely that some extracellular event must trigger the intracellular pathway that leads to cell death, and many researchers have attempted to identify binding partners on the cell surface that could initiate the intracellular process. Several proteins have been suggested and include the laminin receptor [190], stress inducible protein-1 [231], and PrP [20]. Binding of PrP\textsuperscript{106-126} to PrP\textsuperscript{\textalpha} causes direct inhibition of the antioxidant activity of PrP [20]. Treatment of neurons with PrP\textsuperscript{106-126} causes a marked reduction in the resistance of neurons to oxidative stress [94], and a decrease in the activity of other antioxidant enzymes such as Cu/Zn superoxide dismutase [30,219]. In addition, the peptide causes a decrease in the uptake of Cu by neuronal cells [20], and a decrease of Cu incorporation into Cu/Zn superoxide dismutase [21]. It is unclear how these changes are brought about. However, there is evidence that PrP\textsuperscript{106-126} can enter cells [160] and might interact with intracellular proteins in microtubules [28], and aggregates of PrP\textsuperscript{106-126} can cause PrP to become trapped in the aggregates [20].

Interestingly, a subset of antibodies to PrP can also cause in vitro apoptosis, suggesting that inhibition of protein catabolism or its interaction with other proteins might be sufficient to trigger cell death [29]. Recently, it has been shown that similar antibodies injected in vivo can also cause neuronal apoptosis [203]. As antibodies to PrP increase the toxicity of Cu ions to cells, it is possible that interference with the protein’s role in Cu metabolism might be central to the ability of PrP\textsuperscript{106-126} to initiate cell death. However, there is evidence to suggest that the direct effect of PrP\textsuperscript{106-126} on neurons is insufficient to complete its execution. PrP\textsuperscript{106126} also has the effect of compromising the neurons’ ability to deal with stressful conditions [27], and in this way gives neurons a phenotype like that observed for PrP-deficient neurones [26].
Execution of cell death then comes about as a result of this compromised phenotype and one of a number of different stress events such as the production of superoxide [27]. Several studies have identified markers of oxidative stress in the brains of rodents with prion disease. There are increased levels of oxidised lipids in the brains of scrapie-infected hamsters [86]. Another study [87] has shown increased levels of nitrotyrosine and hemeoxygenase-1 in the brains of scrapie-infected mice. These observations imply that significant free radical damage is being generated in the brains of scrapie-infected mice. Additionally, there is evidence for mitochondrial damage in cells from brains of scrapie-infected hamsters and mice [48,128]. These changes include reduction in the activity of mitochondrial enzymes and structural abnormalities in the mitochondria. Other enzymes known to be associated with resistance to oxidative stress, such as catalase and glutathione-S-transferase, show increased expression [128].

Taken together, these results suggest that oxidative stress is involved in the pathology of prion diseases. It is possible that oxidative damage to the brain in scrapie might be a result of damage to the mitochondria, which can generate superoxide. However, the measured level of oxygen radicals detected with dichlorofluorescein in mitochondrial fractions from the brains of scrapie-infected mice was not greatly increased above that of controls [112]. Reactive oxygen species such as superoxide are generated by microglia and the implication of this is that microglia cause damage in the brain of scrapie mice. In vitro studies have demonstrated that microglia can mediate the cell death caused by PrP<sup>Sc</sup> or PrP106-126 [27,78,155,156]. However, microglia activation has also been postulated to be a response to neuronal damage rather than the cause of it. Even if neuronal damage was the sole cause of the microglial response (which is unlikely given the complexity of cell-cell interactions), then the microglial activation is still likely to cause significant production of toxic substances to trigger neuronal apoptosis.

In summary, the neurotoxic mechanism involved in prion disease remains unresolved. However, it is clear that PrP<sup>Sc</sup> accumulation in TSE-affected brain largely precedes development of other changes – i.e. spongiform change and astrogliosis, for which PrP serves as a signal for proliferation [17,18,22,25,88,229]. At the ultrastructural level, apparently normal looking neurons secrete PrP<sup>Sc</sup>, which later fibrillizes and becomes neurotoxic [104], and in vitro experiments highlight some possible intermediate steps.

As mentioned, several synthetic peptides which form amyloid fibrils, e.g. PrP106126 [19] and PrP118-135 [179], induce apoptosis in a dose-dependent manner. PrP106-126 may exert its pro-apoptotic characteristics via disruption of mitochondrial membranes with subsequent release of cytochrome-c and caspase activation [175]. Next, intracellular Ca<sup>2+</sup> concentration rises and another family of proteases, calpains, are activated. In contrast, Bounhar et al. [14] found that PrP may serve as an anti-apoptotic factor protecting neurons in vitro from Bax-induced apoptosis. Removal
of four of five octarepeats (codon 51-91 of PrP) or D178N and T183A PRNP mutations completely abolished this effect. It is apparent that a functional gain of PrP (as PrP$_{TSE}$) may result in changes of anti-apoptotic into pro-apoptotic properties of PrP, which is vaguely reminiscent of changes of anti-oncogenic into oncogenic functions of p53 protein (also involved in apoptosis).

Moreover, there is no consistent correlation between PrP$_{TSE}$, pathology and neuronal death. Following transmission of BSE from cattle to mice, Lasmezas et al. [127] found two lines of BSE-infected mice, one with PrP$_{TSE}$ and typical TSE pathology (vacuolation and astrogliosis), and a second without PrP$_{TSE}$ but with prominent apoptosis of neurons. These observations suggest that PrP$_{TSE}$ is not directly responsible for apoptosis.

However, in another experiment, where neuronal PrP was depleted in NFH-Cre/MloxP transgenic mice, after early spongiform change had developed following scrapie neuroinvasion, Collinge’s group [151] found a reversal of spongiform change, while PrP$_{TSE}$, released probably from glial cells, steadily accumulated. These PrP$_{c}$-depleted Tg mice also became disease-free; thus, the lack of neuronal PrP$_{TSE}$ and/or PrP$_{c}$ protected them from the final prion-related neuronal degeneration. In contrast, in transgenic mice in which PrP transgene was exclusively expressed in astrocytes under the control of GFAP promoter, spongiform change and neuronal decay were observed following scrapie infection [103].

In an independent experiment, the highest densities of apoptotic cells were observed in those neuroanatomical areas in which spongiform change is minimal or absent – viz. retina and cerebellum [120]. Thus, apoptosis and the presence of PrP$_{TSE}$ may not only be uncoupled under specific experimental conditions but may be directly (not mediated through PrP$_{TSE}$) linked to scrapie infectivity.

Neurons may only degenerate and eventually die through a limited number of pathologic pathways. Cellular necrosis is caused by a sudden brain insult,
leading to destruction of the entire cell, the remnants of which attract inflammatory cells. Apoptosis is a programmed molecular process following a “suicidal” stimulus, which leads to a hierarchical gene response. Apoptotic cells, in contrast to necrotic cells, attract no inflammatory response. As the relative lack of classical immunological response in TSE-affected brain is paradigmatic for the whole group of these diseases [31], neurons, by definition, should die by apoptosis and not necrosis.

A determinant for apoptotic cell detection is the time over which neurons die. Even when the number of neurons in dorsal lateral geniculate nucleus (dLGN) following intraocular inoculation drops from over 22,000 to less than 2,000 [101], the number of apoptotic nuclei detected by TUNEL method is low [70]. Because apoptotic cells are most readily detected in highly structured neuronal systems such as the retina and hippocampus, what is observable in less structured regions may represent only a minute proportion of all apoptotic events occurring in TSE-affected brains.

As an evolutionarily ancient cellular response to intra- and extracellular noxious stimuli, autophagy may precede or co-exist with apoptosis, and the process may be induced by apoptotic stimuli [38,227]. Furthermore, the level of autophagy may define the sensitivity of a given neuronal population to apoptotic

Fig. 9. Neuronal autophagic vacuoles in an early stage of development. Note that sequestrated cytoplasm is of high electron density (arrow). SV – synaptic vesicles. A hamster brain infected with the 263K strain of scrapie. Original magn. × 12,000; bar = 200 nm
stimuli, which may underlie the phenomenon of “selective neuronal vulnerability”. Thus, autophagy and apoptosis are often interwoven [40].

Cellular autophagy is a physiological degradation process employed, like apoptosis, in embryonic growth and development, cellular remodelling and the biogenesis of some subcellular organelles – viz. multilamellar bodies [65,91,192]. Autophagosomes coalesce with lysosomes to form degraded autophagic vacuoles, and as in apoptosis, only excessive or misdirected autophagy causes a pathological process. Autophagy is highly enhanced in other brain amyloidoses or conformational disorders [83], Alzheimer’s disease [206], Parkinson’s disease [2], and Huntington’s disease, in which the signal for autophagy is huntingtin [110]. Here, we extend these observations using different models of scrapie and CJD.

**Neuronal autophagy in TSEs**

Data on autophagy in TSEs are very limited, consisting of a few electron-microscopic papers, including reports from our own laboratories [132,136,143-145,199]. The pioneering work was published in Acta Neuropathologica by Boellaard et al. [9]. Our initial experimental approach using the hamster-adapted 263K or 22C-H strains of scrapie...
was subsequently extended by studies of human brain biopsies from patients with sporadic CJD, variant CJD, and FFI [144]. Experimentally infected animal models are widely used because of their relatively short incubation periods that, for mice, range from 16 to 18 weeks, and for hamsters from 9 to 10 weeks for the 263K strain and 24-26 weeks for the 22C-H strain.

**Formation of autophagic vacuoles in TSEs-affected brains**

Autophagic vacuoles are areas of the cytoplasm sequestered within double or multiple membranes (phagophores) of unknown origin; one possible source is the endoplasmic reticulum (Fig. 8). They contain ribosomes, small secondary vacuoles, and occasional mitochondria. Some vacuoles present a homogeneously dense appearance.

We observed neuronal autophagic vacuoles in different stages of formation in the same specimens and our interpretation of the ‘maturity’ of their formation may or may not equate to actual developmental stages. Initially, a part of the neuronal cytoplasm was sequestered within double or multiple membranes (phagophores) and often exhibited increased electron-density (Figs. 9-11). The intracytoplasmic membranes multiplied in a labyrinth-like
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Fig. 11. Section through a myelinated fibre showing formation of autophagic vacuoles (hearts). A hamster brain infected with the 263K strain of scrapie. Original magn. × 8300; bar = 100 nm

manner (Fig. 11). The autophagic vacuoles then expanded and eventually a vast area of the cytoplasm was transformed into a merging mass of autophagic vacuoles. Occasionally, a single large autophagic vacuole was visible. Autophagic vacuoles developed not only in neuronal perikarya but also in neuronal processes, eventually replacing the whole cross-section of affected neurites (Fig. 9, 12). In a few specimens we found round electron-dense structures that we identified as aggresomes. In general, there was little qualitative difference between these two models, although hamsters inoculated with the 263K strain showed a more robust pathology.

Conclusions and hypotheses

One of the major problems of TSEs pathogenesis is the cause of neuronal degeneration with eventual neuronal loss [69,136]. Whether the prion is or is not the pathogen, it is widely accepted that the basic underlying pathological event is the conversion of a normal isoform of prion protein (PrP\(^\text{c}\)) into its pathological misfolded isoform (PrP\(^\text{TSE}\)) [15,51,178, 183], involving an \(\alpha\)-helical to \(\beta\)-pleated sheath (or \(\beta\)-helical) transformation. Using a highly sophisticated mathematical model, Stumpf and Krakauer [210] tried to reason whether PrP\(^\text{TSE}\) causes neurons to die because of neurotoxic effect of PrP\(^\text{TSE}\) (gain of function), or loss of function of PrP\(^\text{c}\). They assumed that if cells die of apoptosis because of neurotoxic gain of function of PrP\(^\text{TSE}\), the cells should die rapidly, and the amount of PrP\(^\text{TSE}\) should be low. Indeed, in both CJD and FFI, there are more apoptotic cells and a lower amount of PrP\(^\text{TSE}\) [107] than in GSS, where the amount of amyloid is vast and the number of apoptotic cells is low [84].

As already mentioned, three types of programmed cell death (PCD) are known: apoptosis, autophagy and swelling of intracellular organelles. Apoptosis has
been discussed already, and in this part we will concentrate on autophagy in regard to TSEs.

Whereas apoptosis in TSE is relatively well understood, autophagy is not. As already mentioned, autophagic cell death or type II PCS is a process used by a cell to remove the bulk of organelles, e.g. during growth and development, but which becomes pathological if too robust or wrongly placed [39]. According to Bursch et al. [39], the appearance of "autophagic vacuoles in dying cells by electron microscopy is taken as condition sine qua non (i.e. an absolute prerequisite) to denote cell death as autophagic/type II PCD". To this end, the mere presence of autophagic vacuoles is relatively well demonstrated in TSE.

There are several uncertainties in our thinking on neuronal autophagy in TSEs. First, autophagy is regarded as a short-term response to nutrient limitations [61], which is, by definition, not the case in slow transmissible diseases like TSEs. However, it seems that autophagy is activated to prevent apoptosis; when autophagy is blocked, apoptosis ensues. On the other hand, when apoptosis is blocked as in Bax/Bak-deficient mice, autophagy is activated as a cell-survival mechanism [148]. However, when all subcellular organelles are self-eaten, the cell eventually dies. An analogous situation was observed when inhibition of autophagosomes and lysosomes was accomplished via targeting of LAMP2 by RNA interference [82]. A dual role for autophagy was envisaged – i.e. protective role against apoptosis and detrimental role in cell death – and both of these are mediated by the same set of ATG genes [61]. Thus, the
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A abundant presence of autophagy in TSEs suggests that neurons either try to escape apoptosis or die by autophagy via one of the programmed-cell death pathways.

A second uncertainty pertains to the relationship between autophagy and the abnormal processing of PrP. The significance of autophagy for controlling the outcome of misfolded PrP\textsubscript{TSE} is unclear. PrP\textsuperscript{c} is entirely translocated into the lumen of the endoplasmic reticulum where the N-terminal signal peptide is cleaved away and the C-terminal GPI is added (Figs. 3-4) [92]. Then, PrP\textsuperscript{c} is transported on the cell surface. The misfolded PrP\textsuperscript{TSE} is localized to the cell surface and the endosomal-lysosomal compartment (Fig. 6); and the conversion of PrP\textsuperscript{c} into PrP\textsuperscript{TSE} presumably takes place somewhere within one or more of these subcellular compartments [13,41,42]. However, misfolded PrP\textsuperscript{TSE} is directed to the aggresomes where, according to the hypothesis put forward by Cohen and Taraboulos [50], it may form a “seed” for seeded-nucleation process of forming more PrP\textsuperscript{TSE}. Then, aggresomes are engulfed by autophagic vacuoles that, in turn, fuse with lysosomes containing PrP\textsuperscript{TSE}, and the process of nucleation may either be initiated or it is perpetuated. It was found that aggresomes may indeed induce the formation of autophagosomes [117].

A third uncertainty is how autophagy contributes to overall pathology underlying TSEs. The hallmark of TSEs is the vacuole which is intracellular “empty” space surrounded by a single or a double membrane. The histogenesis of vacuoles is not well understood and most ultrastructural studies suffer from the inability to ascertain the subcellular organelles from which vacuoles originate; dilated endoplasmic reticulum or mitochondria have been suggested [102]. Our own unpublished work suggests that vacuoles are formed relatively abruptly with no detectable transitional stages [Gibson and Liberski, personal communication]. It is tempting to speculate that vacuolation in TSEs is somehow related to type III PCD characterized by the presence of large, membrane-
bound intracellular empty spaces without the participation of lysosomes. The other option, that tissue destruction by autophagy results in vacuolation (Figs. 13, 14), would imply that autophagic vacuoles should be detected prior to spongiform vacuoles. Indeed, in the terminal stages of sheep scrapie [M. Jeffrey – personal communication] and in human TSEs (CJD, GSS and FFI) [144], little or no autophagy is seen but robust vacuolation is typical if not pathognomic. In contrast, when earlier stages of TSE have been monitored in experimental rodent models, both autophagy and vacuolation have been reported [105,142,145]. Jeffrey et al. [105] have suggested that their presence reflects the robust accumulation of misfolded PrP\textsuperscript{res}, overloading of the neuronal catabolic machinery, and, eventually, bulk removal of damaged neurons via autophagy.

If this scenario is correct, the pathology of TSEs is akin to the reactivation of certain embryonic processes in which bulk removal of cells are present; i.e. remodelling of insect larvae [173]. Nearly 20 years ago, Elizabeth Beck [8] suggested that robust vacuolation like that characteristic of TSEs is present when rats are inoculated with a suspension of cells from normal immature cerebellum. Neurons of inoculated rats not only demonstrated intracytoplasmic vacuoles but, at the ultrastructural level, showed abundant coated pits and vesicles – phenomena of widespread appearance in TSEs.

The other form of neuronal degeneration is neuroaxonal dystrophy [77,131,133,134]. The ultrastructural correlate of NAD is the dystrophic neurite – an axon or dendrite filled with electron-dense inclusions, many of which were recently recognized as small autophagic vacuoles (Fig. 15) [174]. Both immature autophagic vacuoles immunogold-labelled with abs against Cat-D and mature vacuoles containing catepsin were observed within those neurites [174]. As dystrophic neurites are abundant in TSEs [131,141], it is plausible that (macro)autophagy plays a role in neuronal degeneration in TSEs. However, taking into account the evidence that autophagy may prevent

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**Fig. 15.** Two dystrophic neurites filled with numerous autophagic vacuoles (arrows) and lysosomal electron-dense bodies. A hamster brain infected with the 263K strain of scrapie. Original magn. × 30 000; bar = 200 nm
apoptosis, it is also possible that abundant presence of autophagic vacuoles within dystrophic neurites actually reflects neurons struggling to survive in the noxious environment of misfolded PrPSc.

In summary, autophagy certainly does occur in TSE, but its pathogenetic role as a cause of cell death is uncertain. In particular, more research will be necessary to determine the connection, if any, between programmed cell death and the formation of spongiform change.

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