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Neuronal autophagy and aggresomes constitute a consistent part of neurodegeneration in experimental scrapie

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Abstract

We describe here a variety of autophagous forms encountered in the terminal stages of two models of scrapie in hamsters. We also show that the number of autophagic vacuoles increased through the incubation period in the 263K strain scrapie model. In addition, we demonstrated for the first time the presence of aggresomes in the neuronal cytoplasm in scrapie-affected hamster brains. We observed neuronal autophagic vacuoles in different stages of formation in the same specimens. However, this description is based on the subjective grading of the severity of changes, which may or may not equate to the stage of development. First, a part of the neuronal cytoplasm was sequestered by membranes (phagophores) and that part of the cytoplasm confined by membranes often exhibited increased electron density. The intracytoplasmic membranes multiplied in a labyrinth-like manner. The area taken up by autophagic vacuoles expanded and eventually a large proportion of the cytoplasm was transformed into an agglomeration mass of autophagic vacuoles. Occasionally a large but single autophagic vacuole was visible.
Autophagic vacuoles developed not only in the neuronal perikarya but also in neuronal processes, eventually replacing the whole cross-section of affected neurites. Generally, there were few qualitative differences between these two models but hamsters inoculated with the 263K strain had more severe changes.
In a few specimens there were round electron-dense structures that we identified as aggresomes. Aggresomes are not membrane-bound and were found in the cytoplasm of a few neurons.

Key words: *autophagic vacuoles, autophagy, aggresomes, scrapie, prion diseases, transmissible spongiform encephalopathies*

Introduction

Neuronal degeneration belongs to the classical triad of lesions of transmissible spongiform encephalopathies (TSEs) including kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker (GSS) disease and fatal familial insomnia (FFI) in man [5,11,12]. The conversion of the normal cellular isoform of prion protein (PrP^c) into its pathological disease-associated isoform (PrP^d; d, from "disease" or PrP^{Sc}; Sc, from "scrapie") underlies the basic pathology of TSEs. How the accumulation of PrP^d leads to neuronal degeneration is uncertain at the present time but switching off PrP^d accumulation in conditional knock-out mice seems to revert early pathological changes, and eventually prevents the development of the disease [26]. The repertoire of pathways that lead to neuronal

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death is, however, limited and comprises neuroaxonal dystrophy [20], the accumulation of phosphorylated epitopes of neurofilament proteins [18,19] and different forms of synaptic degenerations [2,22,29]. All these pathways lead to neuronal death, presumably disrupting both neuronal metabolism and circuits that generate a pro-apoptotic signal for neurons [2].

It is generally accepted that neurons in TSEs die by apoptosis [9,29] or type 1 programmed cell death (PCD). Neuronal autophagy represents type 2 PCD while type 3 PCD is similar to type 2 but the involvement of lysosomes is, if at all, negligible [22]. To this end, we and others [3,4,20,22,24,13] have demonstrated neuronal autophagy in experimental TSEs in rodents. Recently we also demonstrated autophagic vacuoles in synaptic terminals of human TSEs [29]. Thus, the presence of autophagy in TSEs seems to be well established.

We describe here a variety of autophagous forms encountered in the terminal stages of two models of scrapie in hamsters. Furthermore, we report that the number of autophagic vacuoles increased through the incubation period in the 263K scrapie model. In addition, we demonstrate for the first time the presence of aggresomes in the neuronal cytoplasm in scrapie-affected hamster brains.

Material and Methods

Animals and strains

Hamsters were inoculated intracerebrally with the 263K or 22C-H strains of scrapie using 0.05 ml of a 10% brain homogenate cleared by low-speed centrifugation [17]. These strains are widely used experimental tools primarily because of relatively short incubation periods which, for mice, ranged 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. Appropriate control animals were sham-inoculated with saline. After 11 days post inoculation, 2 scrapie-injected hamsters and 1 control hamster were used. The clinical endpoint was defined when animals developed unequivocal signs of disease – ataxia, tremor, ruffled fur, urine and bowel incontinence (i.e. fur was stained with urine and faeces) and, for the 263K strain, head bobbing – a rhythmic up and down shaking of the head.

Electron microscopy

Animals were anaesthetized with ether and killed by intracardiac perfusion. Following intracardiac perfusion with 2.5% gluteraldehyde and 1% paraformaldehyde in cacodylate buffer (pH 7.2), the brains were removed and rinsed in cold fixatives overnight. Samples [1 mm^3] of the right parietal cortex, hippocampus, thalamus, and corpus callosum were dissected, rinsed in phosphate buffer, post-fixed in 1% osmium tetroxide, dehydrated through a graded series of ethanols and propylene oxide and embedded in Embed (Electron Microscopy Sciences, Ft. Washington, PA). Ultrathin sections were stained with lead citrate and uranyl acetate, and specimens were examined using a Zeiss 109 or Jeol JEM 100 C transmission electron microscope.

Results

Control animals

No autophagic vacuoles were found in sham-inoculated animals.

Scrapie-infected animals

Definition of autophagic vacuoles

Autophagic vacuoles are composed of areas of the cytoplasm (Fig. 1) sequestrated with single, double or multiple membrane (phagophores) originated from the endoplasmic reticulum. Sequestered cytoplasm contains ribosomes, occasionally mitochondria, small secondary vacuoles with vesicles, or present a homogeneous dense appearance.

Autophagic vacuoles in neuronal cytoplasm

Generally, there were few qualitative differences between these two models but hamsters inoculated with the 263K strain revealed more severe changes. We observed neuronal autophagic vacuoles in different stages of formation in the same specimens. However, this description is based on the subjective grading of the severity of changes that may or may not equate to the stage of development. First, a part of the neuronal cytoplasm was sequestered by membranes (phagophores) and that part of the cytoplasm confined by membranes often exhibited increased electron density (Figs. 2, 3). The intracytoplasmic membranes multiplied in a labyrinth-like manner (Fig. 4). The space taken by autophagic vacuoles expanded and eventually a large proportion of the cytoplasm was transformed into an agglome-

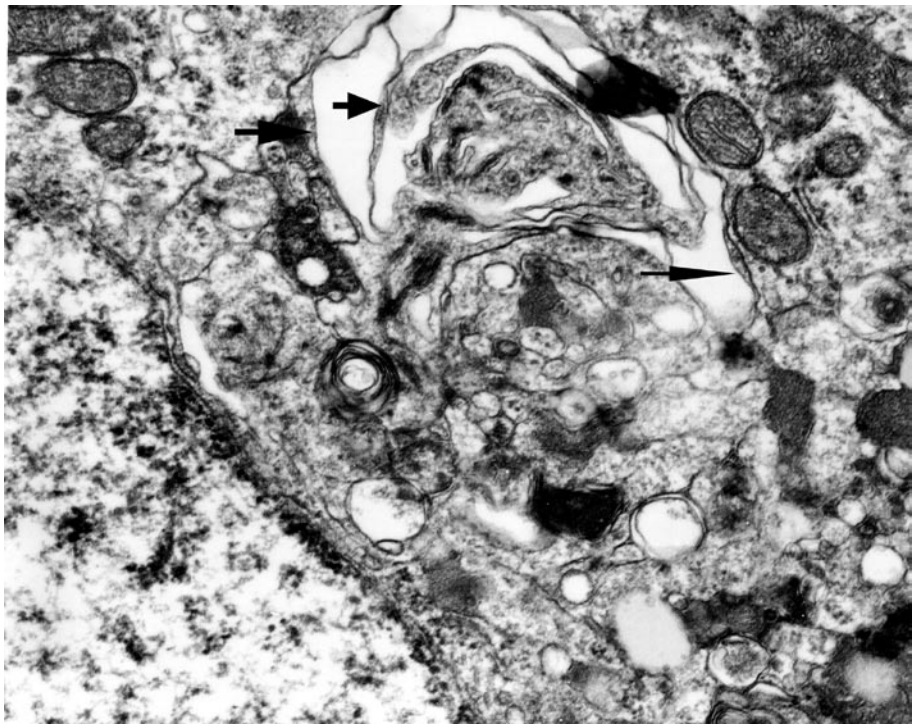

Fig. 1. Large autophagic vacuoles in the neuronal cytoplasm of scrapie-affected hamster brain. 26K strain of scrapie. Note multiple membranes (arrow) lining the vacuoles, original magnification, × 20 000.

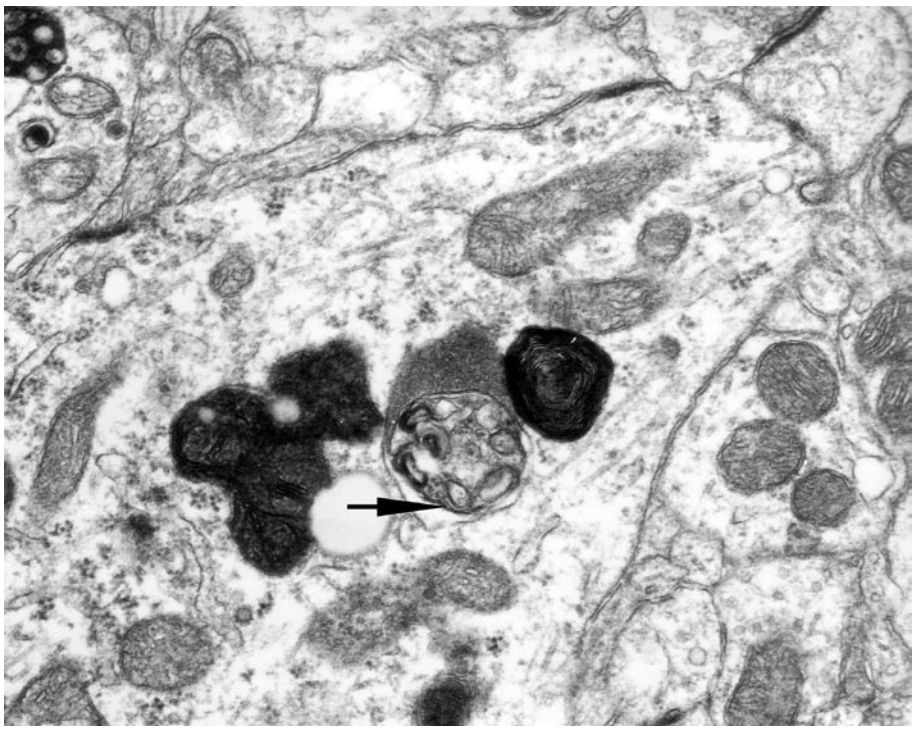

Fig. 2. A small autophagic vacuole (arrow) forming within the cytoplasm of a neuron. 263K strain of scrapie, original magnification, × 20 000. Note the lypofuscin granule in close contact with the vacuole

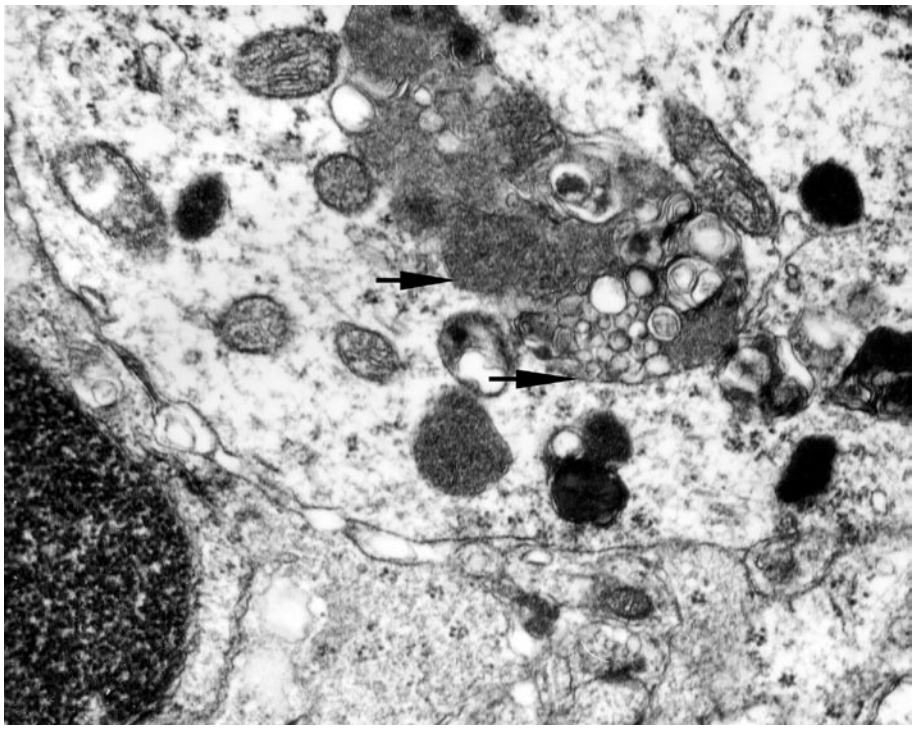

Fig. 3. Larger area of the neuronal cytoplasm is transformed into an autophagic vacuole (arrow). 263K strain of scrapie, original magnification, × 20 000

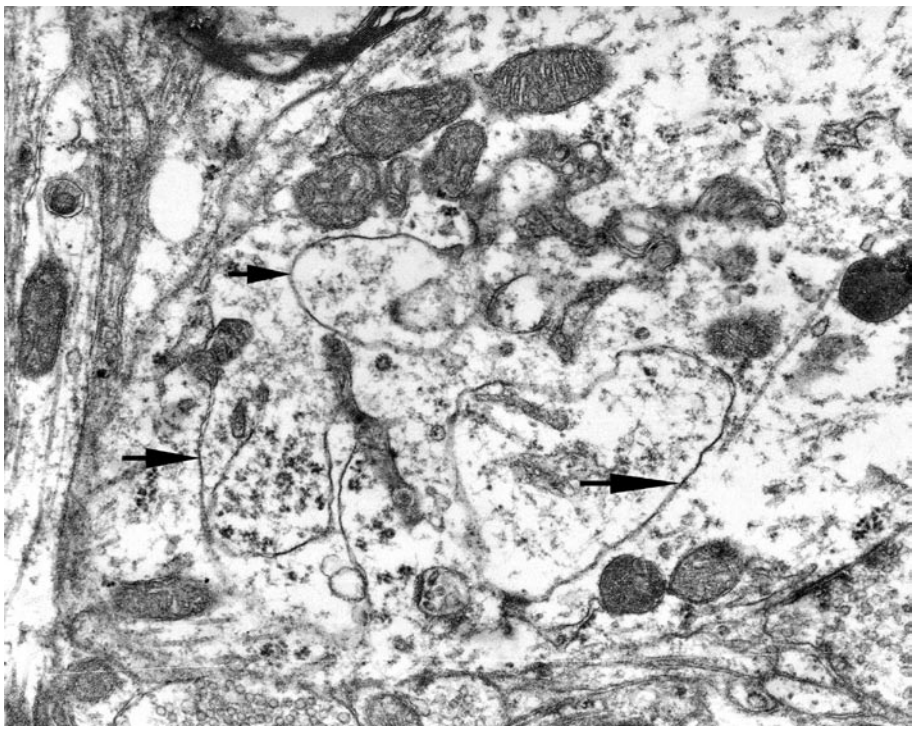

Fig. 4. Phagophores (arrows) in the neuronal cytoplasm of scrapie-affected hamster brain. Note the sequestration of a part of the cytoplasm by phagophores. 263K strain of scrapie, original magnification, × 12 000

ration mass of autophagic vacuoles (Figs. 5, 6). Occasionally, a large but single 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. 7). In the 263K strain of scrapie model, for which the numerical data were available, the number of autophagic vacuoles increased through the incubation period to the highest number at the terminal stage of the disease (Table I).

In a few specimens we also found round electron-dense structures that we identified as aggresomes (Fig. 8). Aggresomes are not membrane-bound and were found in the cytoplasm of a few neurons.

Discussion

We report here that large numbers of autophagic vacuoles may occur in both neuronal perikarya in the brain and in their processes in the terminal stage of experimental scrapie in hamsters and that the number of autophagic vacuoles increased through the incubation period. We propose the following sequence of events: vacuoles start to form in a restricted area of the cytoplasm and then expand finally to occupy a large area of the cytoplasm. However, it is also possible that vacuoles of different shape and size develop simultaneously. The preferred scenario that destruction of neurons by autophagy is initiated from a confined region of the neuronal cytoplasm fits well with the hypothesis that conversion of PrP^c into misfolded PrP^d occurs through a seeded-nucleation mechanism that is a stochastic event [16]. Experiments were initiated to determine how autophagy develops through the incubation period.

Table I. Development of autophagic vacuoles within dystrophic neurites in hamster brains infected with the 263k strain of scrapie

No	DPI	DN	DN/AV	DN/AV%
60	11	18	4	22.2
61	19	28	8	28.57
63	32	39	9	23
71	35	6	2	33.3
70	74	63	30	47.62

No – number of the specimen; DPI – days post inoculation – i.e. incubation period; DN – number of all dystrophic neurites; DN/AV – number of dystrophic neurites containing autophagic vacuoles; DN/AV%, DN/AV – number of dystrophic neurites containing autophagic vacuoles in percentage

Autophagy in TSE was first described by Boellard et al. [4] in experimental scrapie. It was subsequently reported in experimental scrapie [3,20,24] and CJD [20,24]; in BSE transmitted to mice [11,13] and in human CJD [29]. We also observed autophagy in scrapie-affected hamsters but erroneously labelled it "membranous whorls" [17]. Jeffrey et al. [13] suggested that autophagy reflects abnormal PrP^d processing within the lysosomal and endosomal pathways.

While apoptosis in TSE is relatively well understood, autophagy is not [29] and studies on autophagy are limited. Autophagy or type II PCD is a process used by a cell to remove the bulk of organelles, such as during growth and development, and which becomes detrimental if too severe [6]. Neither the molecular phenomena that initiate the autophagy nor molecular markers for the identification are known. According to Bursch et al. [6] the appearance of "autophagic vacuoles in dying cells by electron microscopy is taken as the condition sine qua non (i.e. an absolute prerequisite) to denote cell death as autophagic/type II PCD". Thus the presence of autophagic vacuoles in TSE as demonstrated in this paper is evidence for the occurrence of type 2 PCD.

The presence of aggresomes in the scrapie-affected hamster brain is the first indication that these subcellular organelles develop *in vivo* as demonstrated *in vitro* [7,25,27]. Conversion of mostly α-helical normal cellular isoform of prion protein (PrP^c) to its misfolded pathological isoform PrP^d is accepted as the basic pathogenetic event. According to Cohen and Taraboulos [7], abnormally processed PrP^d is directed to aggresomes, where it may form a seed for a "seeding-nucleation" process of formation of more PrP^d [10,16]. Then, aggresomes are engulfed by autophagic vacuoles that, in turn, fuse with lysosomes containing PrP^d and the process of nucleation may either be initiated or perpetuated. Indeed in neuroblastoma cells transfected *in vitro* with three different mutant PrP^ds (V203I, E211Q and Q212P), all three mutant proteins accumulated in aggresomes [27].

Aggresomes are cytoplasmic organelles that develop as a cellular response to misfolded proteins [14]. They are not formed randomly but rather at the microtubule-organizing centre (MTOC) by an ordered process and then remain stable. Aggresomes may be induced not only as a response to misfolded proteins but also by overexpression of proteins normally de-

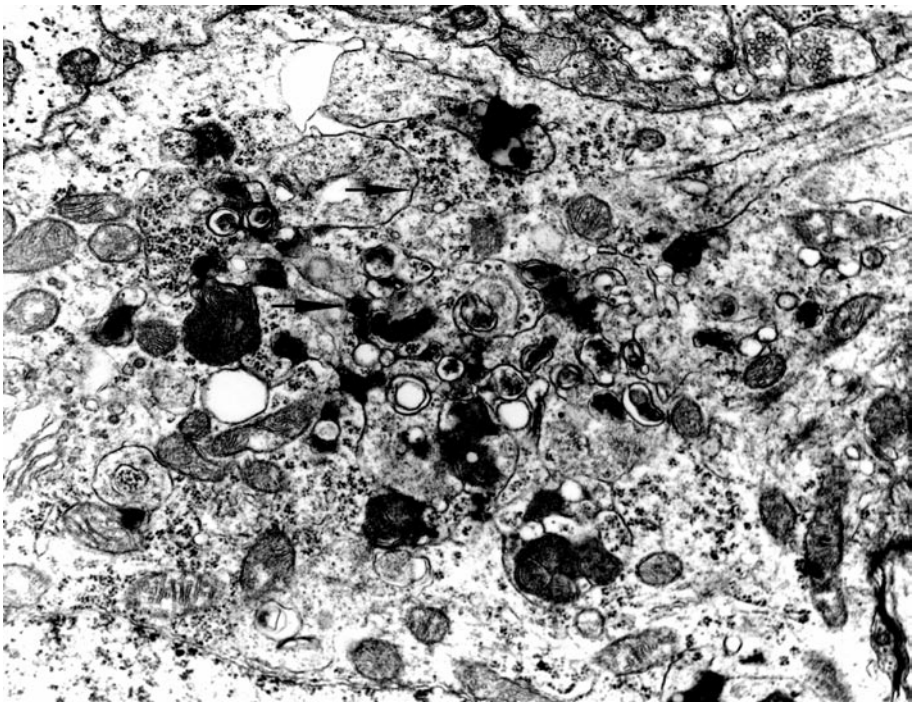

Fig. 5. A large area of the neuronal cytoplasm is transformed into an autophagic vacuole. Membranes lining the vacuole are visible (arrows). Note the diversity of the subcellular structure of the vacuole's components and some cellular debris within the vacuole. 263K strain of scrapie, original magnification, × 20 000.

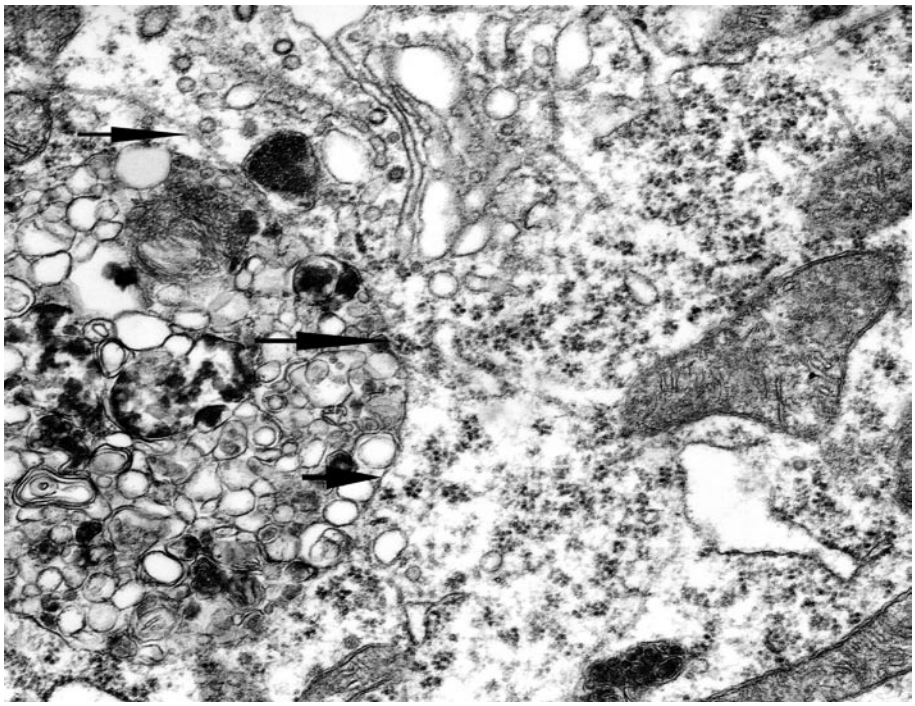

Fig. 6. A large area of the neuronal cytoplasm is transformed into a complex autophagic vacuole; phagophores are marked with arrows. Note the diversity of the subcellular structure of the vacuole's components and some cellular debris within the vacuole. 263K strain of scrapie, original magnification, × 20 000

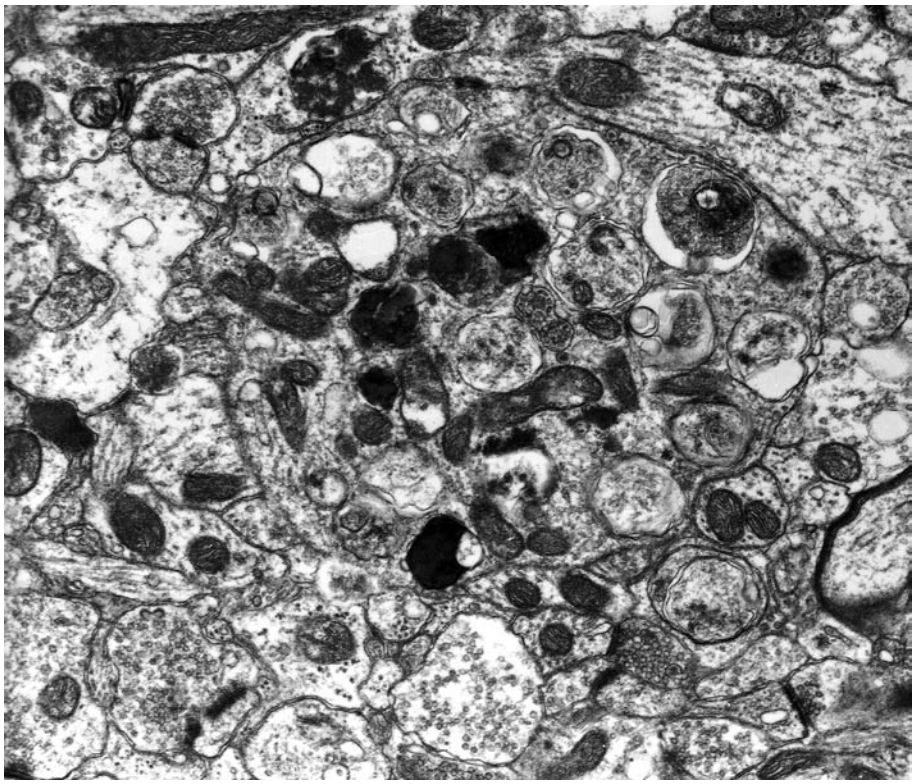

Fig. 7. A cross-section through the neuronal process containing a large number of autophagic vacuoles and a single multivesicular body in the centre. 263K strain of scrapie, original magnification, × 12 000

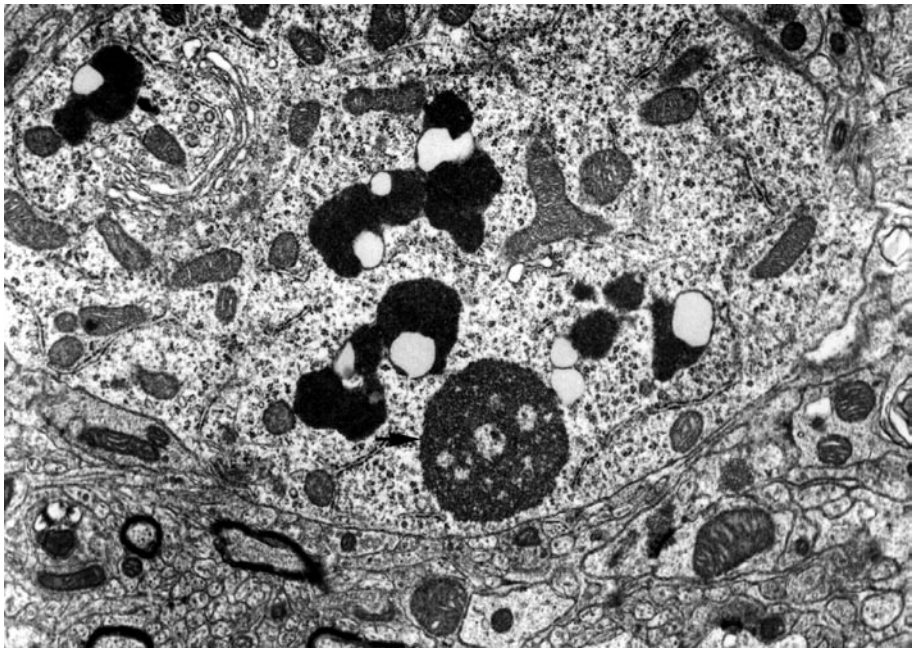

Fig. 8. An aggresome (a large round structure in the centre labelled with an arrow) within the cytoplasm of the neuron in scrapie-affected hamster brain. 263K, original magnification, × 12 000

graded by the proteasome or by the inhibition of the latter structure [30]. Various misfolded proteins, presenilin-1, cystic fibrosis transmembrane conductance regulator (CFTR) [14] and parkin [15] that, like PrP^d, are misfolded proteins are associated with "conformational disorders" that are directed to aggresomes. There are further similarities. Overloading of the proteasomal cellular "quality control" system for protein degradation *in vitro* with excess of abnormally folded mutated protein, ΔF 508CFTR and D177N PrP^d, respectively [14, 25] leads not only to aggresome formation but also to a predominance of deglycosylated species of both proteins. All these data suggest that, like other proteins which are triaged by the proteasomal cellular "quality control" system for protein degradation, misfolded PrP^d is channelled to aggresomes. This system is overwhelmed and this prevents proper degradation of PrP^d and results in accumulation of this neurotoxic protein. More interestingly, aggresomes are likely to trigger an alternative mechanism of protein degradation – autophagy.

How autophagy contributes to the overall pathology underlying TSEs is unclear. The hallmark of TSEs is the vacuole, which is an 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 of clear detection of subcellular organelles from which vacuoles originate – dilated endoplasmic reticulum or mitochondria have been suggested [13]. We also considered 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, suggests that vacuoles and autophagy are disconnected in time; i.e. one may see either autophagic vacuoles or spongiform vacuoles and the former should precede the latter. Indeed, in scrapie-affected sheep (M. Jeffrey – personal communication) and in human CJD [23], in which it is only possible to study the terminal stage of disease by electron microscopy, no or very little autophagy is seen but well developed vacuolation is typical if not pathognomic. By contrast, in rodent models in which one may (at least in principle) observe a sequence of events, both autophagy and vacuolation have been reported [13,21,24]. Along these lines, Jeffrey et al. [13] suggested that their presence reflects the marked accumulation of misfolded PrP^d, overloading of the neuronal catabolic machinery and, eventually, bulk removal of damaged neurons by autophagy.

If this sequence of events is correct, the pathogenesis of TSEs may reflect the reactivation of primeval embryonic processes in which bulk removal of cells occurs, e.g. remodelling of insect larvae [28]. To this end, Beck [1] suggested that well developed vacuolation, not unlike that characteristic of TSEs, may be elicited following inoculation of rats with a suspension of cells from normal immature cerebellum. Neurons of inoculated rats not only demonstrated intracytoplasmic vacuoles but, at the ultrastructural level, showed an abundance of coated pits and vesicles, phenomena commonly found in TSEs [8].

In summary, autophagy takes place in TSEs but its role, especially its relationship with the accumulation or removal of misfolded PrP^d, is hypothetical at the present time. More research is necessary, especially to show whether there is, or is not, a link with programmed cell death and the formation of spongiform change.

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