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Neuronal autophagy in experimental Creutzfeldt-Jakob's disease

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Summary. We report an experimental model of Creutzfeldt-Jakob's disease (CJD) in mice leading to the formation of giant autophagic vacuoles (AV) in neurons of the cerebral cortex. These AV appear at the end of the incubation period (4–6 months post-inoculation), together with spongy changes and clinical symptoms. Autophagy, a process of intracellular digestion of cell constituents by the lysosomal compartment, is known in many cell types, where it plays a role both in the physiological turnover and in pathological processes and is involved in protein metabolism. The same also occurs in neurons. Here autophagy is known to occur in the normal state and leads to residual bodies called lipofuscin granules. An increase in lipofuscin is known to occur in human and experimental CJD. Therefore, an increase in autophagy and in AV can be expected. In our experimental model, the activation of neuronal autophagy may be related to an alteration in neuronal protein metabolism.

Key words: Creutzfeldt-Jakob's disease – Prion – Lipofuscin – Neuronal autophagy – Lysosomes

Sequestration and subsequent digestion of the damaged portion of cytoplasm following local intracellular injury have been postulated [1]. Where this occurs, there are changes that indicate degradation of the cytoplasmic remnants without any evidence of damage to the remainder of the cell. Hruban et al. [21] referred to this process as focal cytoplasmic degradation to distinguish it from total cytoplasmic degeneration and cell necrosis.

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Apart from being involved in these clearly pathological events, the process of autophagy [10] takes part in the physiological turnover of cell constituents [16]. This process is linked to the lysosomal compartment, either by fusion of autophagosomes with pre–existing lysosomes, known as "classical autophagy", or by uptake of substances into lysosomes in a pinocytosis-like process called "microautophagy" [33]. Neuronal cells do not divide during their lifespan and accumulate varying amounts of so-called age pigment or lipofuscin in their cytoplasm [17, 36, 38]. The lysosomal nature of lipofuscin granules has been demonstrated in liver cells [13] and in neurons of the CNS [7, 27]. Gradual transition of phagolysosomes into lipofuscin has been reported [7], thus supporting the concept that lipofuscin granules are residual bodies containing the products of previous autodigestive processes.

In human and experimental Creutzfeldt-Jakob's disease (CJD) the amount of neuronal lipofuscin is increased [14, 22, 23]. Therefore, an increase in neuronal autophagic processes in this disease can be suspected, but has not so far been proved. We report the results of an investigation into this question carried out on experimental CJD in mice which had been induced according to the method of Tateishi et al. [41, 42].

Material and methods

Eighteen NZW mice and one crossbred mouse were included in the test group (Table 1). Four were used in the first passage. These animals were inoculated intracerebrally (i.c.) into the right occipital lobe with 0.02 ml of 10% human CJD brain homogenate, and killed 632–652 days later because the incubation period of the first passage is longer than that in subsequent passages [42].

Twelve further animals were inoculated similarly with 10% mouse CJD brain homogenate. These animals were allowed to live for 1 to 6 months after the inoculation. The clinical symp-

Table 1. Test animals

No.	Months	Strain	Spongiosis	Autophagy
a intracerebral inoculated with Creutzfeldt-Jacob's disease (CJD) mouse homogenate				
1	6	NZW	+	+
2	6	Crossbred	+	+
3	6	NZW	+	+
4	5	NZW	+	+
5	5	NZW	+	+
6	4	NZW	+	+
7	3	NZW	+	−
8	3	NZW	−	−
9	2	NZW	−	−
10	2	NZW	−	−
11	1	NZW	−	−
12	1	NZW	−	−
b Intraperitoneal inoculated with CJD mouse brain homogenate				
13	10½	NZW	+	+
14	10½	NZW	+	+
15	10½	NZW	+	+
No.	**Days**	**Strain**	**Spongiosis**	**Autophagy**
c Intracerebral inoculated with human CJD homogenate (1st passage)				
16	652	NZW	+	+
17	652	NZW	+	+
18	632	NZW	+	+
19	632	NZW	+	+

toms of the disease become apparent 4 to 6 months after inoculation [42]. To avoid secondary complications arising from the refusal of food or water, the animals were not allowed to live with signs of illness for longer than a few days. In the three remaining test animals, mouse CJD brain homogenate was inoculated intraperitoneally (i.p.) to avoid direct interference with the brain as a possible source of increased autophagic processes. Because of the longer incubation period associated with this route of transmission [42], the animals were killed 10.5 months after inoculation.

Eighteen animals of various strains were used as controls (Table 2): twelve NZW mice were inoculated i.c. in the same way as the test animals but with brain homogenates taken from a patient with olivo-ponto-cerebellar atrophy (OPCA) or with human lymphocytes. Lymphocytes were inoculated, because they have an infectivity similar to that of brain homogenates in a CJD patient [43]. They were killed 1−6 months after inoculation. One DDD mouse was inoculated with normal mouse brain and killed 23 months after inoculation. Three senescence-accelerated mice (SAM) and two CBA/J mice were kept longer under the same feeding condition but without any treatment to disclose ageing effect. The former with short life span [40] and the latter, one of the amyloid-prone strains [6], were killed in old age. These and the DDD mouse were used as age-matched controls for the four test animals in the first passage.

The animals were anesthetized with ether and killed by cardiac perfusion of a mixture of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. Brain specimens were obtained from the cortex of both the right and left parietal lobes. The tissue was postfixed in osmium tetroxide, block-stained with uranyl acetate, dehydrated in a graded series of ethanol and embedded in Epon. Semithin sections were cut and stained with toluidine blue. Ultrathin sections were stained with lead acetate and examined in a Zeiss EM9 electron microscope.

Additional samples from the cortex of both cerebral hemispheres were embedded in paraffin and stained with hematoxylin and eosin and the periodic acid-Schiff (PAS) reaction. Because of the infectivity of the tissue, no material was used for enzyme reactions.

Specimens of the cerebellar vermis and cerebellar hemispheres from three of the test animals (nos. 1, 4, and 5) were also examined by electron microscopy. In one case (no. 13), the thalamus was cut by chance and examined additionally.

Results

As the most conspicuous changes were found in the animals infected by the intracerebral route in subsequent passages, these results will be described first.

Light microscopy

In semithin sections, the cerebral cortex appeared to remain unaltered during the first 3 months. From month 4, patchy spongiosis, which was sometimes confined to the white matter, was found in the test animals. At this stage, spherical intracytoplasmic PAS-positive bodies were found in a few neurons in paraffin-embedded cortical tissue.

Electron microscopy

This revealed very few neuronal alterations in the test animals in the first 3 months. The perikarya were sometimes shrunken or cleared in their peripheral regions. Small or sometimes larger myeloid bodies could be found in neurons of test as well as control animals.

From the 4th month, membrane-bound vacuoles could be found in the perikarya of a few neurons in all the test animals. No difference in their distribution was seen between the two cerebral hemispheres (Table 1). These vacuoles contained ribosomes and coated vesicles (Figs. 1a, 2b), membrane-bound compartments (Figs. 1c, 2a), and tightly packed small spherical bodies (Figs. 3, 4b). The latter were also found in some places scattered in the neuronal cytoplasm (Fig. 3). These vacuoles were separated from the surrounding cytoplasm by a membrane which was usually single but in places double, and sometimes strongly osmiophilic (Fig. 1c). They were interpreted as autophagic vacuoles (AV) because of the cytoplasmic derivates they contained, most of which could be identified as ribosomes and endoplasmic reticulum (ER) (Figs. 1b, 2b). The AV seemed to enlarge by apposition (Fig. 2b). In some neurons, however, this process of growth may not have taken place, and here we saw many small AV in the affected neuron (Fig. 3). The neuronal cytoplasm in general revealed no signs of damage. Apart from patchy spongy changes in

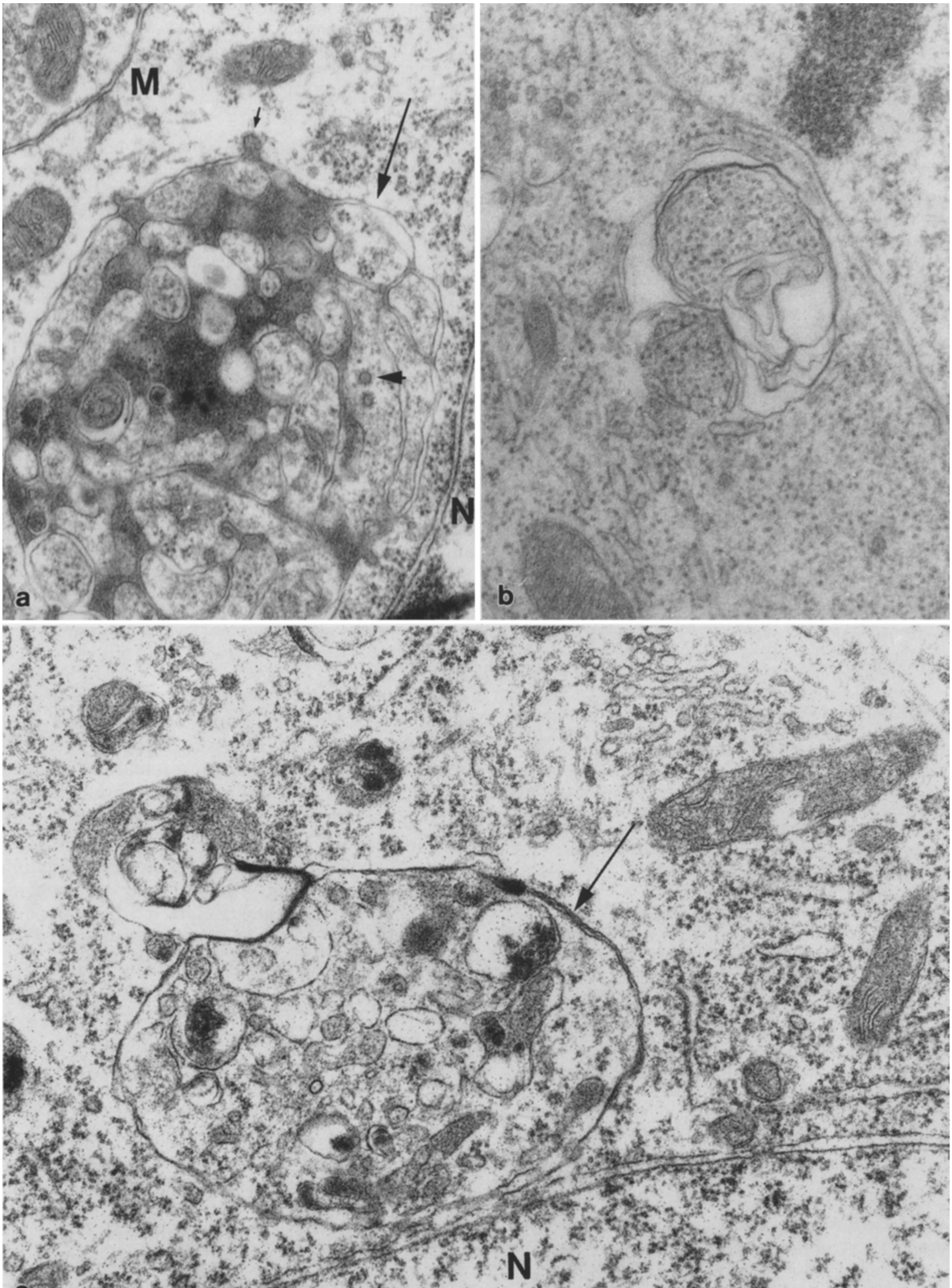

Fig. 1a–c

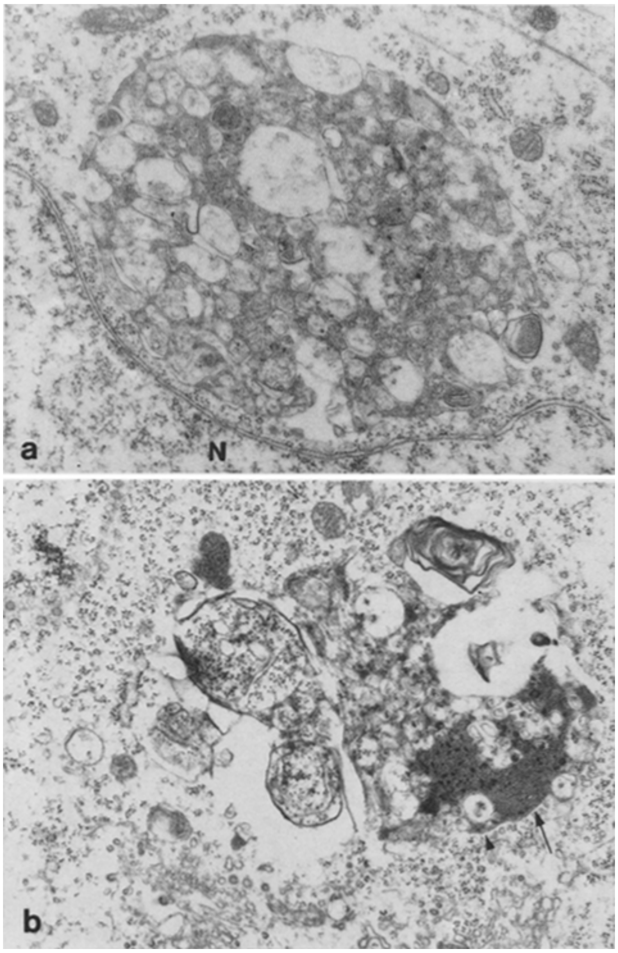

Fig. 2. a Neuronal AV with many membrane-bound bodies filled with detritus. The membranes are well defined. *N*: Nucleus. Test mouse, 6 months after inoculation i.c. **b** AV filled with detritus and membranous material. Lipofuscin (*arrow*) consisting of amorphous pigment and membranes (*arrowhead*). Attached to this is a presumably newly formed AV filled with ribosomes and endoplasmic reticulum. Test mouse 6 months after inoculation i.c.

Fig. 1. a Autophagic vacuole (AV) with membrane-bound compartments filled with ribosomes (*arrow*) and coated vesicles (*arrowhead*). *Small arrow*: Coated pit; *N*: Nucleus; *M*: cell membrane. Test mouse 6 months after inoculation i.c. **b** Presumably newly formed AV filled with ribosomes and surrounded by a multilamellated membrane. *N*: Nucleus. Test mouse 6 months after inoculation i.c. *c* AV with partly bilamellar (*arrow*) lipid membrane. The membrane connects the large AV with a smaller one (apposition). Both are filled with membrane-bound bodies. *N*: Nucleus. *G*: Golgi field. Test mouse, 4 months after inoculation i.c.

the neuropil there were, here and there, vacuoles in neuronal perikarya. Those neurons, however, which contained AV did not show other perikaryal vacuoles. In these cells the cytoplasm was well preserved with often very well-developed Golgi complexes with coated pits.

Transitional stages between these AV and unit-membrane-limited bodies containing clumps of granular dense material intermingled with membranous structures were often observed (Fig. 4a). Corresponding to these findings, increased numbers of lipofuscin complexes, which were often huge, were found in the perikarya of some neurons but the bulk of these cells contained only very few, small pigment granules, as usually seen in the neurons of these animals. The number and appearance of the lysosomes showed no obvious change. We failed to find any phagosomes in astrocytes: these structures were just occasionally seen in oligodendrocytes and microglia.

AV of the kind described were also found in some of the cortical neurons of the three test animals injected intraperitoneally (Table 1), although they were scanty in number (Fig. 4b). In one of these cases we had the opportunity to examine the thalamus and scattered intraneuronal AV like those in the cortex, were found.

We also found neuronal AV in the four animals of the first passage, although only very rarely (no more than 1 – 3/ultrathin section). There were no giant AV but several smaller ones in few neurons (Fig. 5). Spongiform changes in the neuropil were patchy (Table 1). The neuronal lipofuscin content did not exceed that of the age-matched cases.

In the cerebellar cortex of three test animals injected i.c. and killed 4 to 6 months after inoculation, the granule cells and the large perikarya of the Purkinje cells proved to be unremarkable and without obvious signs of autophagy. In the controls of groups a and b (Table 1) there were slight signs of neuronal autophagy, such as multivesicular bodies or in bulk autophagy of cell organelles, but no AV as seen in test animals. These signs of normal turnover of cytoplasmic components were more numerous in the age-matched controls. Here we found small AV such as in the corresponding test group in one neuron of no. 18 (Table 2).

Discussion

Successful experimental transmission of CJD to mice has been reported [25, 41]. Our results concur with these reports with regard to the spongiform changes, which at the light microscopic level, are often more severe in the white matter than in the neuropil [41, 42].

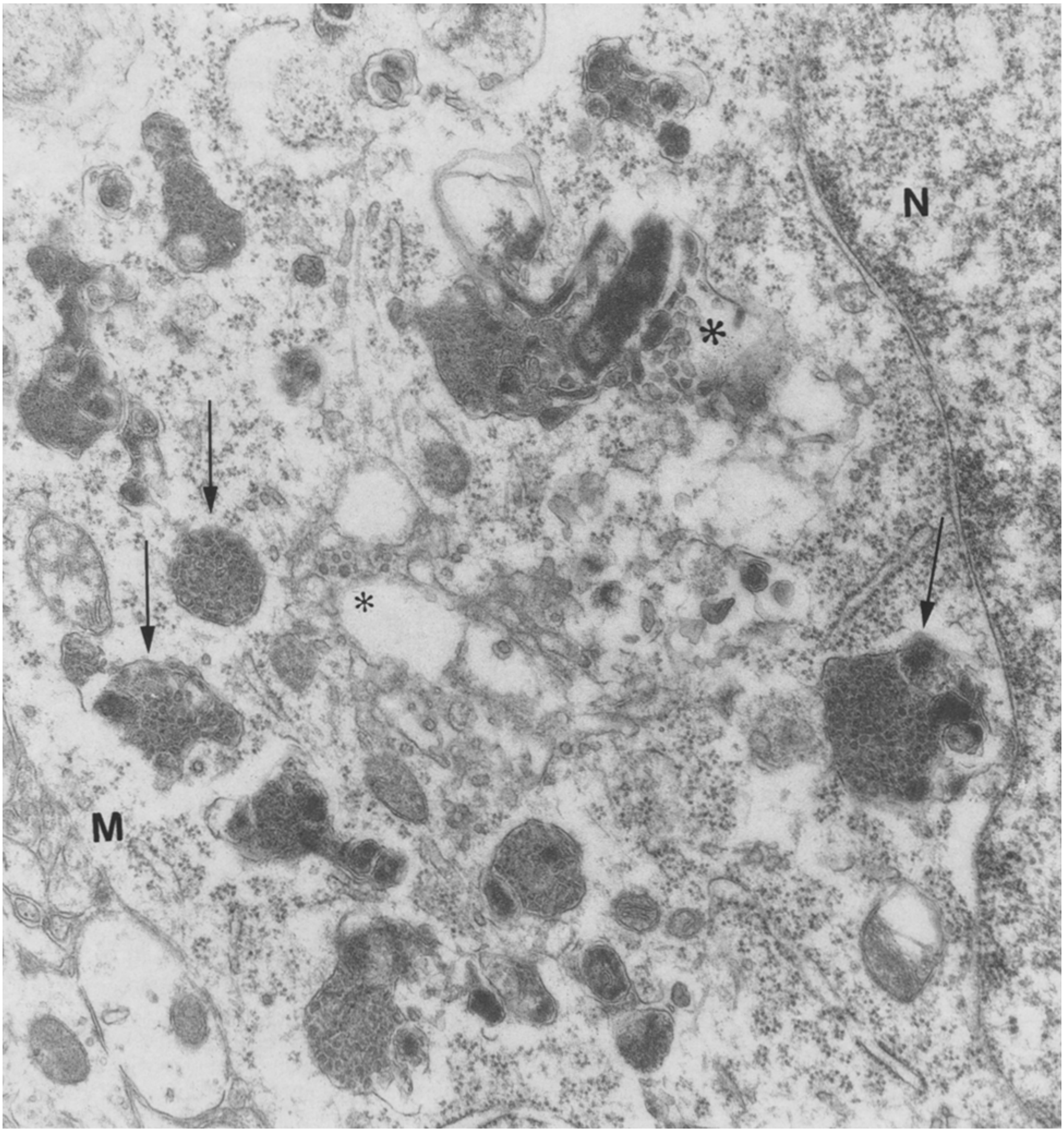

Fig. 3. Neuronal perikaryon with several vacuoles representing transitional forms between AV and residual bodies. In addition, there are many small spherical membrane-bound bodies (*asterisk*) scattered in the cytoplasm and lying tightly packed in some of the vacuoles (*arrows*). *N* Nucleus; *M*: cell membrane. Test mouse 6 months after inoculation i.c.

Autophagy in normal neuronal cytoplasm is well known as an indicator of catabolic activity under physiological conditions [4, 7, 15, 20, 24, 29, 35]. In accord with these facts we, too, have seen signs of autophagy such as larger myeloid bodies or in bulk autophagocytosis of cell organelles in the controls. In no case, however, we have seen large AV as seen in

Fig. 4. a Autophagic material with signs of transformation to lipofuscin. The latter shows amorphous material mixed with membranes and lipid droplets. On the right another AV (*arrow*) with a multilamellated membrane. Both AV contain small membrane-bound particles. *N*: Nucleus; *G*: Golgi apparatus. Test mouse 6 months after inoculation i.c. **b** Cortical neuron in test animal after inoculation i.p. Giant vacuole filled with small membrane-bound bodies. *N*: Nucleus; *M*: cell membrane

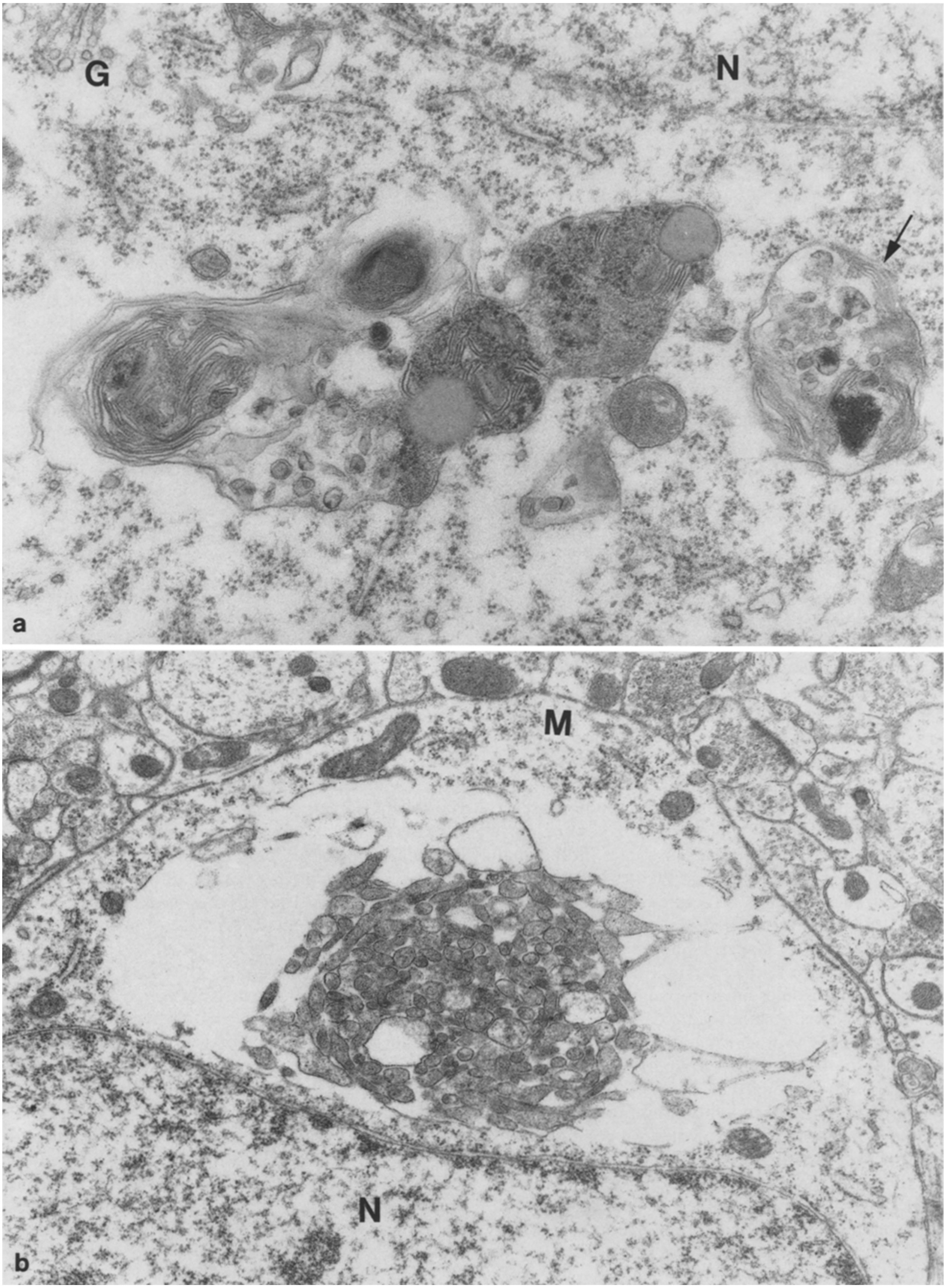

Fig. 4 a, b

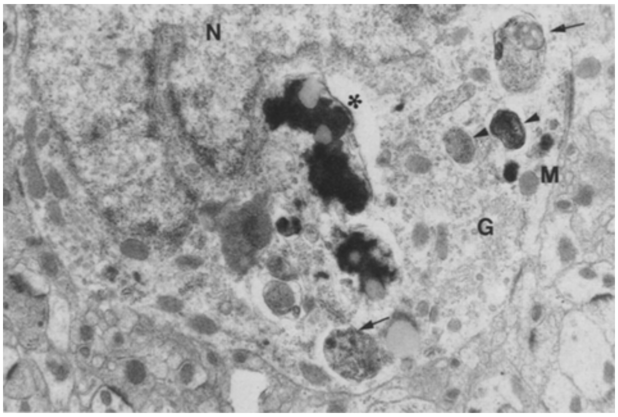

Fig. 5. Cortical neuron in first passage animal. AV (*arrows*), vacuoles containing degraded material (*arrowheads*), and lipofuscin granules (*asterisk*). *N*: Nucleus; *G*: Golgi apparatus; *M*: cell membrane with synapses

Table 2. Control animals

No.	Months	Inoculated with:	Strain
a Intracerebral inoculated			
1	1	Human brain OPCA	NZW
2	1	Human brain OPCA	NZW
3	2	Human brain OPCA	NZW
4	2	Human brain OPCA	NZW
5	2	Human lymphocytes	NZW
6	3	Human lymphocytes	NZW
7	3	Human lymphocytes	NZW
8	4	Human lymphocytes	NZW
9	4	Human lymphocytes	NZW
10	5	Human lymphocytes	NZW
11	5	Human lymphocytes	NZW
12	6	Human brain OPCA	NZW
13	23	Normal mouse brain	DDD
b No inoculated			
14	10		SAM
15	18		SAM
16	18		SAM
17	21		CBA/J
18	21		CBA/J

the test animals. In one old animal, an age-matched control animal of the first passage group (no. 18) we found small AV as seen more frequently in the experimental animals of this group in one neuron.

Neuronal autophagy has also been described under experimental conditions: associated with retrograde degeneration [2, 3], X-irradiation [18], trimethyltin [5] and microtubule poisons such as colchicine [44] and vinblastine [18, 45]. Autophagy of neurons or other cell types can, in general, be activated by amino acid deprivation, but has not previously been mentioned in connection with infectious processes (Siglen, personal communication).

The nomenclature of these membrane-bound areas is based on the absence or presence of lysosomal enzymes as phagosomes or phagolysosomes. Following Marzella and Glaumann [26] and Pfeiffer [32] we have designated the vacuolar structures described here, clearly containing neuronal cytoplasmic components at least in their early stages, as autophagic vacuoles (AV) without regard to their possible enzyme content. The role of lysosomes in autophagic processes of neurons is so well established [7, 18], as it is in parenchymal cells of other organs [12, 19, 28, 30], that we decided not to employ enzymatic methods with this highly infectious material.

Neuronal AV are frequently found in mice of the subsequent passages after an incubation period of 4–6 months post inoculation i.c. They are more scarce 10.5 months after infection by the i.p. route, and they are very scarce, and also smaller, in the first passage after an incubation period of about 23 months following i.c. inoculation. Thus, there seems to be a correlation between the duration of the incubation period and the degree of autophagy, i.e., the number of neurons containing AV.

The AV are filled with cytoplasmic components and their remnants in various stages of degradation. The most striking contents, however, are linear membranous structures and small membrane-bound bodies (Figs. 2a, 4b). These AV are distinguished by these structures from autophagosomes occurring under other conditions. They may be the hallmark of these giant phagosomes.

Many vacuoles represent transitional stages of degradation between AV and lipofuscin (Figs. 2b, 4a). This is not surprising since autophagy, being a lysosomal process, leads to the formation of residual bodies, i.e., lipofuscin [7, 27].

Altmann [1] and Hruban et al. [21] considered autophagy to be a pathological process restricted to the sequestration of local areas of damaged cytoplasm to avoid complete cell necrosis. The presence of large AV reported here would fit well with this concept. However, autophagy is involved in the lysosomal pathway of intracellular protein degradation and reutilisation under physiological conditions [11, 31] and is a random process [16]. Pathologically altered autophagy can predominantly be caused by alterations of intracellular amino-acid levels [33]. In our model this could mean an alteration of protein metabolism. The AV in our study appeared at about the same time as the clinical symptoms and spongiosis: in the hamster scrapie model, this point in time coincides with the climax of PrP formation, but not with that of infectivity, as the latter occurs earlier [8, 9]. There seems to be no doubt as to a connection of neuronal autophagy and the administration of the CJD agent several months earlier. Whatever this may be, a relationship can be assumed to exist between an altered neuronal protein metabolism, possibly caused by the processing of PrP, and the resulting neuronal autophagy in this experimental model.

Referring to the question of whether there may be an autophagic processes in human CJD, it should be held in mind that autophagy is an energy-dependent process [37, 39]. The half-life of autolysosomes in neurons is not known, but it has been established in other organs, such as liver and kidney. The average half-life of AV in liver cells has been shown to amount to about 9 min for protein [34]. Therefore, it is unlikely that AV will be found in CJD autopsy material. Here we can only expect to find their footprints. According to Kirschbaum [23], an increase in neuronal lipofuscin in CJD has often been reported.

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