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Subependymal plaques in scrapie-affected hamster brains – why are they so different from compact kuru plaques?

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Abstract

We report here routine thin-section and immunogold electron microscopic studies on diffuse plaques in scrapie-affected hamster brains. These plaques were not discernible by routine H&E staining. Ultrastructurally, plaques were recognized as areas of low electron density containing haphazardly-oriented fibrils, but not as stellate compact structures typical of mouse scrapie models; hence we labelled them "loose plaques". Following immunohistochemistry at the electron microscopy level, fibrils within plaques were heavily decorated with PrP-conjugated gold particles. Loose plaques were located beneath the basal border of the ependymal cells and around blood vessels in the adjacent subependymal neuropil. When dystrophic neurites containing electron-dense inclusion bodies, some of them autophagic vacuoles [59], were seen within the plaque perimeter, they always remained PrP-negative. Some microglial cells were observed in close contact with PrP-positive plaques, and secondary lysosomes within these cells were heavily decorated with gold particles.

Key words: *prion diseases, transmissible spongiform encephalopathies, scrapie, amyloid plaques.*

Introduction

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases or transmissible brain amyloidoses, are neurodegenerative diseases caused by a still incompletely characterized infectious agent that, according to the most widely, albeit not exclusively [1,6,48], accepted theory, is designated a "prion" [35,39,57]. The accumulation of PrP amyloid is a crucial event in TSE pathogenesis [1,18]. This protein is derived from a host-encoded cell-surface sialoglycoprotein known as PrP^c. The disease-specific form is a misfolded isoform of PrP^c known as PrP^{TSE} or PrP^d (where "d" stands for disease). PrP^d may be formed by a process of nucleation or a template-directed polymerization from its normal cellular isoform PrP^c. During this conversion, PrP^c changes its conformation from predominantly an α-helical structure into β-pleated or β-helical form [56]. Thus, TSEs are diseases of protein misfolding, diseases of protein conformation [10] or, according to Beyreuther and Masters' [5] poetic term, "protein cancers".

PrP^d, also known as "PrP-amyloid", is deposited extracellularly in the form of amorphous or primitive plaques, classical (kuru) plaques and as congophilic angiopathy [38]. Kuru plaques are observed in all

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cases of Gerstmann-Sträussler-Scheinker disease (GSS), the majority of cases of kuru, and a small proportion of cases (approximately 10-15%) of Creutzfeldt-Jakob disease (CJD) and chronic wasting disease in cervids [20,21,36,49]. Amyloid plaques also constitute a hallmark of neuropathology of scrapie [24] and CJD models in rodents [31]. Furthermore, hydrated or hydrolytic autoclaving methods coupled with PrP immunohistochemistry enables the demonstration of different types of PrP^d accumulation in all TSEs studied thus far and has became the current standard among tests to diagnose TSE at the tissue level [22,30,33,34,45,58,64].

In electron microscopy, extracellular PrP-amyloid plaques are confined to the CNS and consist of different proportions of amyloid fibrils, dystrophic neurites and microglial cells [47]. The latter cells may function as amyloid scavenger cells because intra-microglial PrP^d may be detected in lysosomes in areas without amyloid fibrils: this suggests these cells take up or accumulate excess PrP^d. Most amyloid plaques in TSE which are visible at the light microscopy level are characterized by a compact stellate core and relative paucity of dystrophic neurites and microglial cells; these are called "kuru" plaques or, if they merge as in GSS, multicentric plaques [3,11,51]. Some plaques do not exhibit, however, such compact architecture but rather loose tissue structure with amyloid fibrils still discernible within it.

We report here routine thin-section and silver-enhanced immunogold electron microscopic studies on diffuse plaques in scrapie-affected hamster brains. While such plaques were first observed almost 20 years ago by Wiley et al. [62], this is the first detailed ultrastructural study of those plaques, which differ considerably from compact "kuru" plaques encountered in scrapie-affected mouse brain. It is thus plausible that the morphology of PrP deposits is influenced not only by a particular strain of the agent but also by species-specific local brain microenvironment [17].

Material and Methods

Animals strain, experimental procedures

All procedures were performed according to rules promulgated by the National Institutes of Health. Two groups (5 animals in each group) of outbred, 6-week-old golden Syrian hamsters were inoculated intracerebrally with 0.05 ml of a 10% brain suspension of either the 263K or 22C strain of scrapie (kindly provided by Dr. Richard Kimberlin, SARDAS, Edinburgh, UK and Dr. Richard Carp, IBR, NY, USA, respectively). These strains are widely used experimental tools primarily because of their relatively short incubation periods, which ranged from 9 to 10 weeks for the 263K strain and 24-26 weeks for the 22C-H strain. Two control hamsters from the same colony were sham-inoculated with saline. The clinical endpoint was defined when animals developed unequivocal signs of disease – ataxia, tremor, ruffled fur, urine and bowel incontinence (fur was stained with urine and faeces) and, for the 263K strain, head bobbing – a rhythmic up and down shaking of the head.

Electron Microscopic Examination

Following deep ether anaesthesia and injection of 1 ml of heparin into the heart, terminally ill hamsters (5 animals inoculated with the 263K and 5 animals inoculated with the 22C-H strain) and 2 age-matched control animals from each group were sacrificed by intracardiac perfusion with 100 ml of 1.25% glutaraldehyde and 1% paraformaldehyde prepared in cacodylate buffer (pH 7.4) followed by 50 ml of 5% glutaraldehyde and 4% paraformaldehyde. To this end, an animal was pinned on its back, the thorax was opened wide around the sternum and the abdominal part of the aorta descendens was clamped. Then, the right auricle was cut and a tube was inserted through the incisure in the left ventricle into the aorta ascendens. Perfused carcasses were kept at 4°C for 2 hours before the brains were removed and rinsed in cold fixative overnight. Samples (1 mm^3) of the right parietal cortex and adjacent corpus callosum, the CA1 region of the hippocampal formation, the thalamus and the subventricular regions were dissected, rinsed in phosphate buffer, postfixed 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 with Philips 300, JEM 100 CDX and Zeiss EM 109 transmission electron microscopes.

Immunogold procedures

The immunogold methods employed for ultra-structural localization of PrP^d were as previously

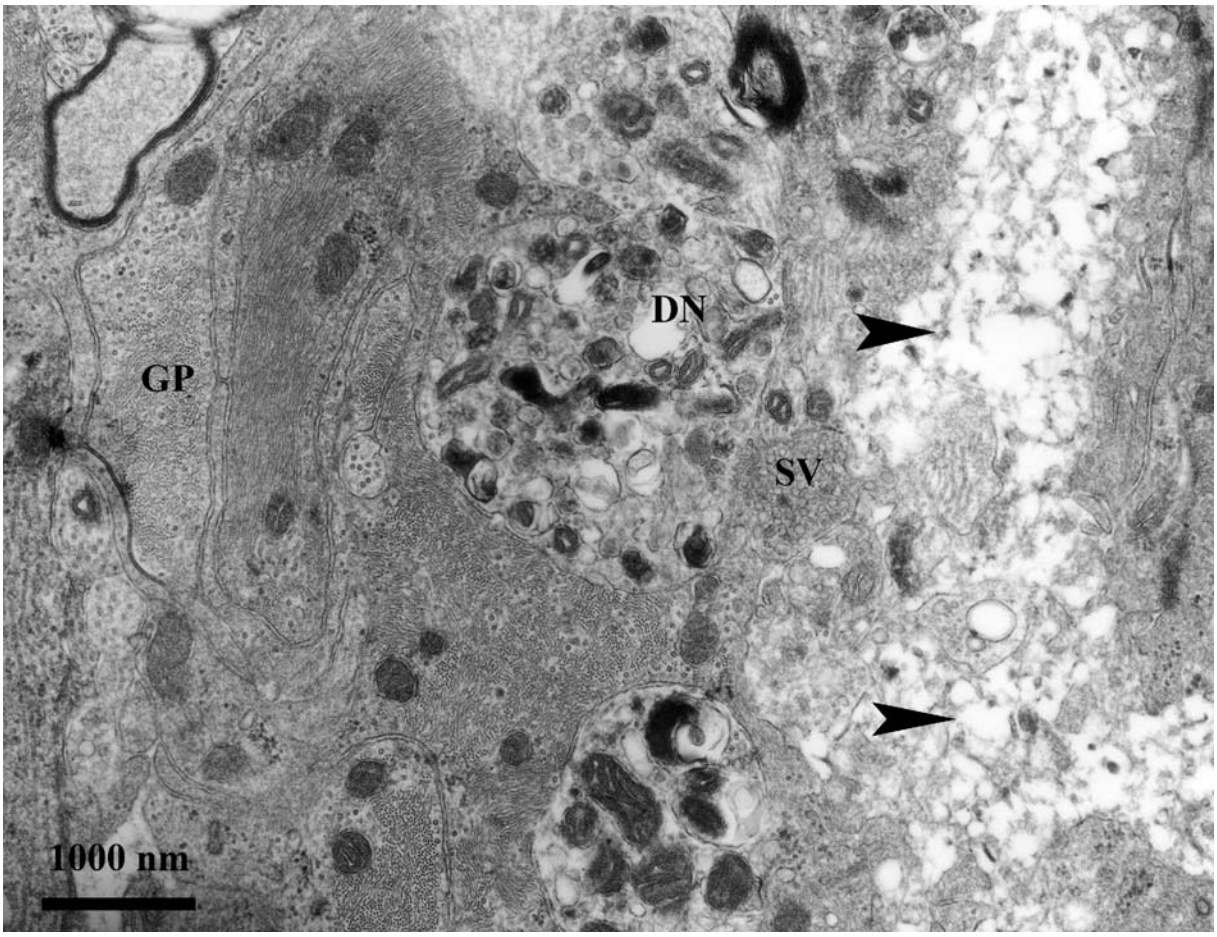

Fig. 1. Low power electron micrographs of "loose plaque" (arrowheads) in scrapie-affected hamster brain. Note that plaques developed in the subependymal region; numerous glial processes (GP) are visible in the vicinity as well as dystrophic neuritis (DN) and some synaptic terminals containing synaptic vesicles (SV)

described [41]. Briefly, these methods are as follows. 65-80 nm sections were taken from blocks previously identified as containing accumulations of PrP^d after light microscopy of stained 1 µm sections. Sections placed on 400 mesh nickel grids were etched in sodium periodate for 60 minutes or in potassium methoxide DMSO for 15 minutes. Endogenous peroxidase was blocked and sections de-osmicated with 3% hydrogen peroxide in methanol for 10 minutes. Antigen expression was enhanced with formic acid for 10 minutes. Primary antibodies (1B3 and 1A8, kindly supplied by Dr. James Hope, MRC & BBSRC Neuropathogenesis Unit, Edinburgh, Scotland) were then applied in 1:100 dilution or 1:400, respectively, in incubation buffer for 1 hour. After rinsing, sections were incubated with Extravidin 1 nm colloidal gold diluted 1:10 in incubation buffer for 1 hour and silver enhanced. Grids were postfixed with 2.5% glutaraldehyde in PBS and counterstained with uranyl acetate and lead citrate.

Results

The subependymal region from control hamsters was entirely normal. By light microscopy and semi-thin (1 mm) sections, discrete PrP^d-immunopositive amorphous plaques were observed in both the 263K and 22C-H models in the subependymal region but not in the deep brain neuroparenchyma (data not shown). No differences were observed using either 1B3 or 1A8 antibodies. These plaques were discernible neither by routine H & E staining nor by Congo red staining; thus, they are, by definition, not amyloid plaques. Ultrastructurally, plaques were recognized as areas of low electron density containing haphazardly-oriented fibrils (Fig. 1) and, following the immunogold

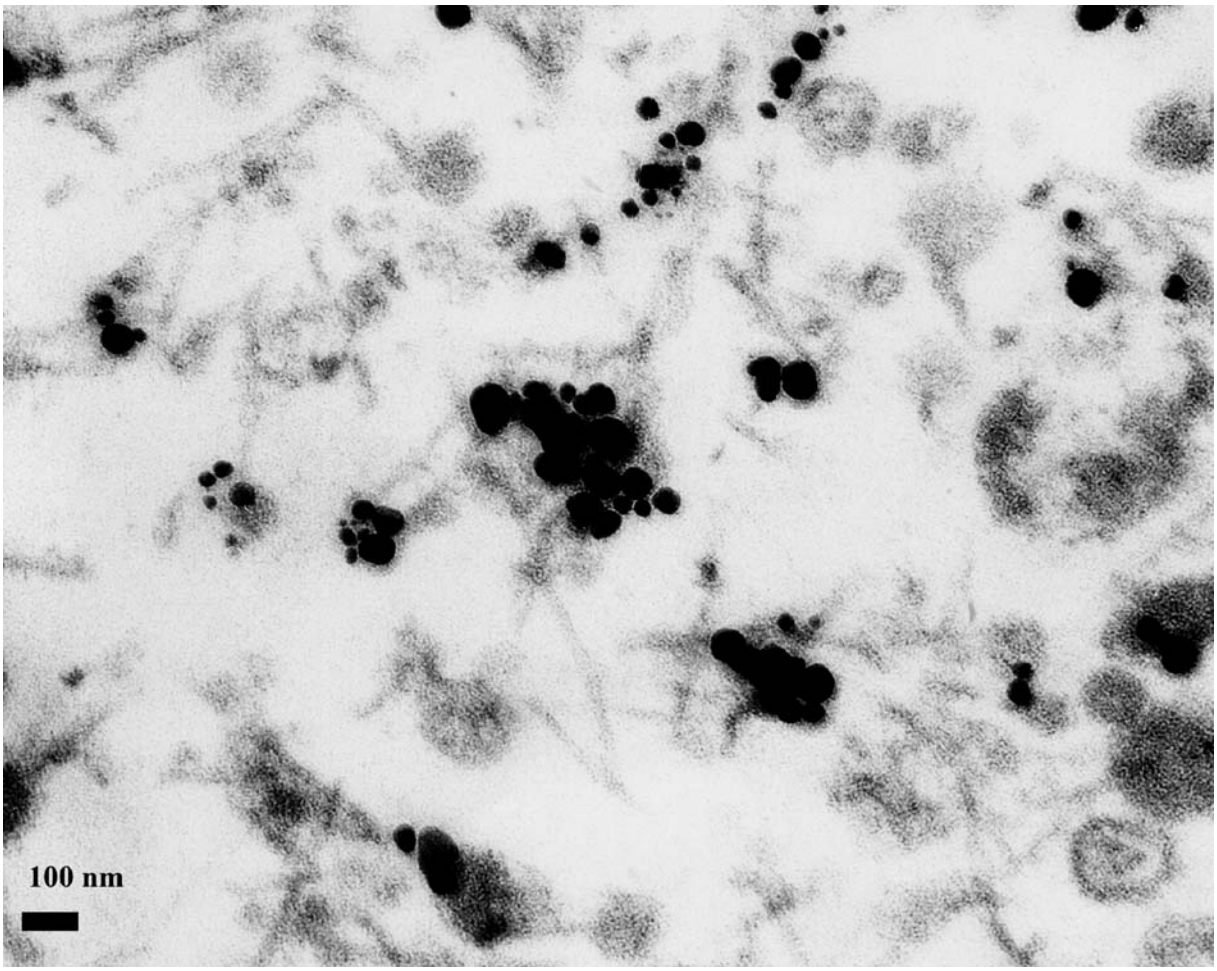

Fig. 2. An electron micrograph of "loose plaque" in scrapie-affected hamster brain. Note amyloid decorated with silver-enhanced gold particles

procedure, heavily labelled with PrP^d-conjugated silver-enhanced gold particles (Fig. 2), but not as stellate compact structures designated "kuru-plaques". These plaques were located beneath the basal border of the ependymal cells and around blood vessels in the adjacent subependymal neuropil, the fine structure of which was clearly recognizable. When dystrophic neurites containing electron-dense inclusion bodies were seen within the plaque perimeter, they always remained PrP^d-negative (Fig. 3). Some microglial cells were observed in close contact with PrP-positive plaques and secondary lysosomes within these cells were heavily labelled with gold particles (Fig. 4). In these two scrapie models neither stellate plaques nor PrP-immunodecorated dendrites were observed.

Neuronal processes containing tubulovesicular structures (TVS), 25-37 nm virus-like particles specific for all TSEs at the level of thin-section electron microscopy and regarded as disease-specific [42,48], were observed in the vicinity of loose plaques (Fig. 5). Furthermore, TVS-like particles were seen attached to amyloid fibrils floating within loose plaques (Figs. 6, 7).

Discussion

We have demonstrated that PrP^d-immunoreactive amyloid plaques in scrapie-affected hamster brains are located mostly beneath the ependymal border. They exhibit not the compact structure of the kuru plaque but rather randomly-oriented amyloid fibrils deposited within expanded extracellular space with an admixture of dystrophic neurites and microglial cells. We thus confirm the plaque pattern first described by DeArmond [13-15]. In addition to their observations, we showed that microglial cells not only

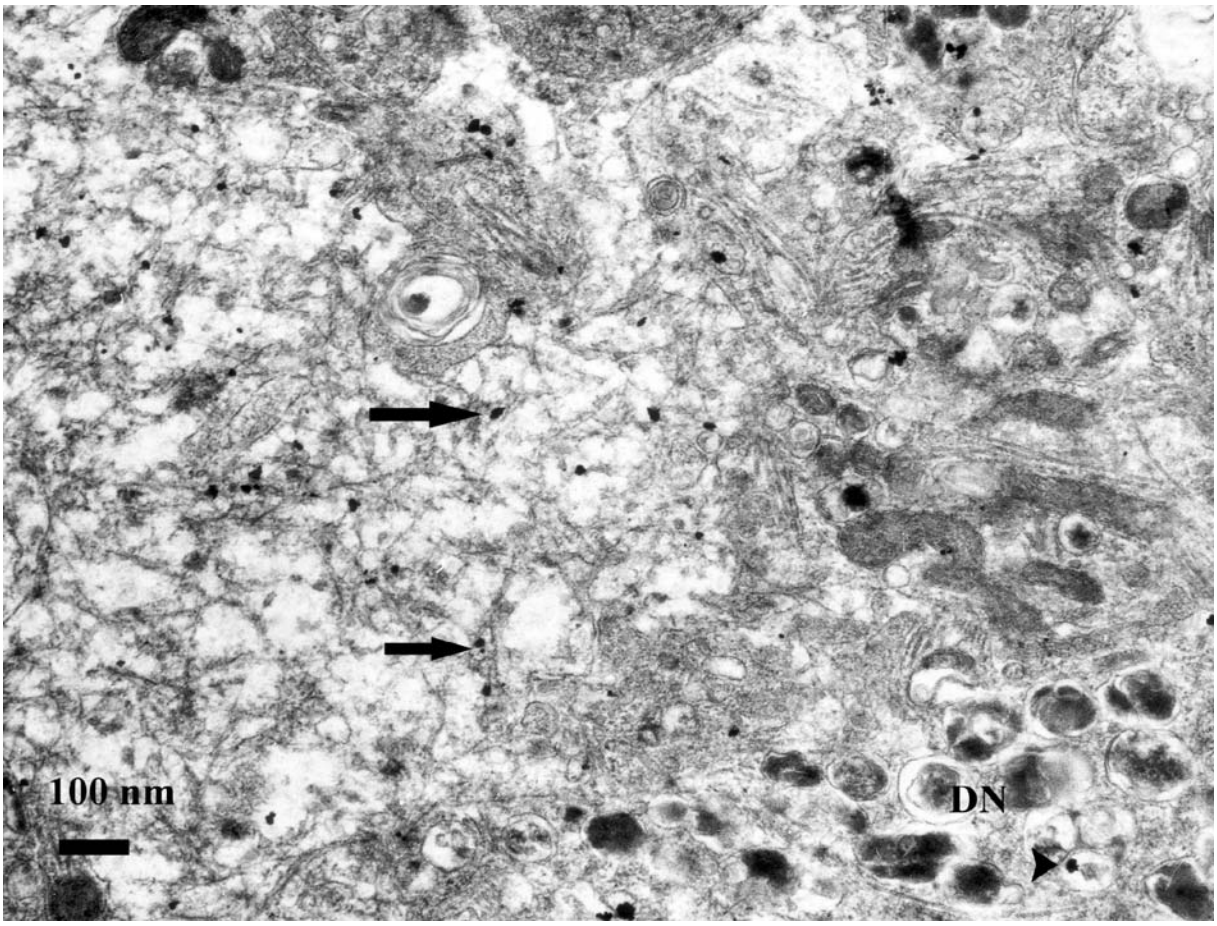

Fig. 3. An area of loose plaque heavily decorated with silver-enhanced gold particles (arrows). Note that a dystrophic neurite (DN) is largely free from immunogold deposits (only one immunogold-silver enhanced deposit on the neurite – arrowhead). Original magn. × 12 000

contain cellular debris but are also heavily labelled within secondary lysosomes [2,8,9,40,50,53]. Overall then, PrP^d-subependymal plaques in scrapie-affected hamster brains are different from those observed in mouse brain infected with the 87V strain of scrapie agent and also different from typical kuru plaques of human TSE [24-29,36]. The sequence of events which leads to formation of the amyloid plaque is not yet clear. In the 87V mouse model, immunogold electron microscopy demonstrated PrP^d on the cell membrane before fibrilization, which suggests that PrP^d is initially shed into the extracellular space where the amyloid fibrils are formed [25,27]. The assembly of fibrils may be a subsequent and entirely spontaneous process of nucleation, and indeed recent *in vitro* experiments suggest that PrP^d is generated from its precursor (PrP^c) as a result of protein-protein (PrP^d – PrP^c) (reviewed in: [10]) interaction (nucleation rather than template-directed polymerization). How these *in vitro* experiments reflect the *in vivo* situation of the amyloid plaque formation has not yet been established but it is tempting to suggest that the microglial cell may provide a microenvironment for such a conversion [3,51]. The exact role of this cell is, however, also totally unclear [63]. The presence of PrP^d within the lysosomal-endosomal system has been clearly demonstrated by numerous investigators including ourselves [2,14,15,40,53], but it is subject to ambiguous interpretation. It may merely reflect the phagocytosis of PrP^d. However, earlier work on *in vivo* generation of PrP^d from PrP^c suggests that PrP^d, like all other brain amyloids including Aβ of Alzheimer's disease and α-synuclein of Parkinson's disease and dementia with Lewy bodies, is generated somewhere along the lysosomal-endosomal pathway, and the presence of PrP^d within this compartment strongly

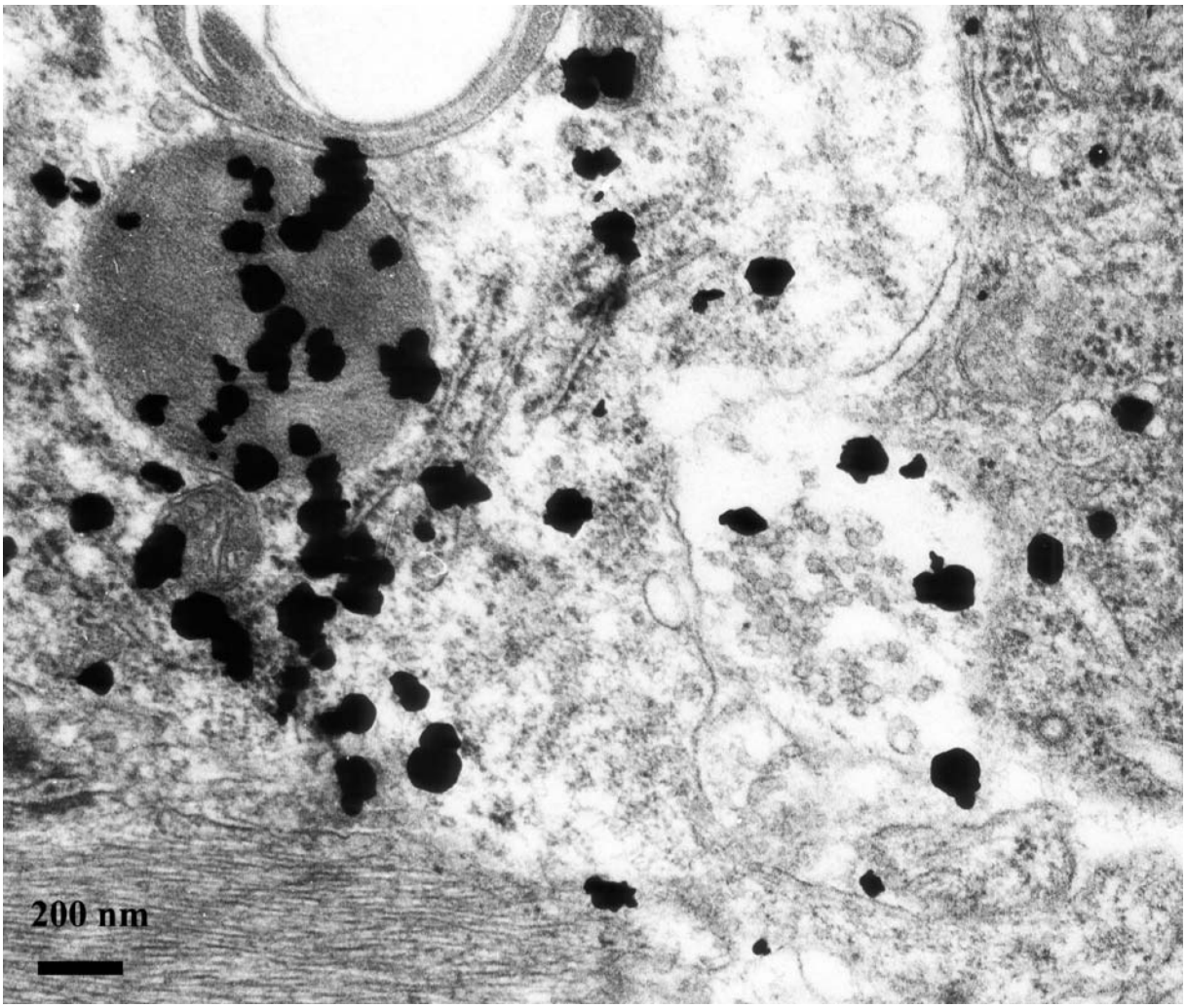

Fig. 4. Secondary lysosome from a microglial cell; the lysosome is heavily decorated with immunogold. Original magn. × 50 000

suggests that the microglial cell is the cell in which the conversion may take place [3,54,55].

The concept of scrapie as a brain amyloidosis first envisaged by Gajdusek [19] has evolved for the last two decades since the description of various forms of brain amyloids in GSS [49] and the demonstration of amyloid fibrils in all TSE. Like amyloidoses in general and brain amyloidoses in particular, accumulation of PrP^{d} follows the same characteristic pattern. Mutations in a gene (*PRNP* in humans; *Prnp* in lower mammals) which encodes for amyloid precursor (PrP^{c}) cause PrP-amyloidosis in familial forms of a disease (GSS or CJD) and different mutations are linked to diverse phenotypic expression of these diseases (for review: [32]). The same amyloid accumulates in sporadic cases in which no mutation in the gene encoding for PrP is found; this is probably accomplished via poorly understood post-translational conformational modifications. Furthermore, overexpression of either the mutated or wild-type gene encoding for PrP^{c} in transgenic animals leads to the development of "spontaneous" disease which in many aspects resembles TSE [16,23,52,61]. However, if transgenic mice are produced with one copy of a transgene (thus, without over-expression), no disease is observed [4]. Collectively, these results point to a crucial role of PrP as "scrapie amyloid" and, by the same token, encourage further studies on the pathogenetic mechanisms by which it is formed.

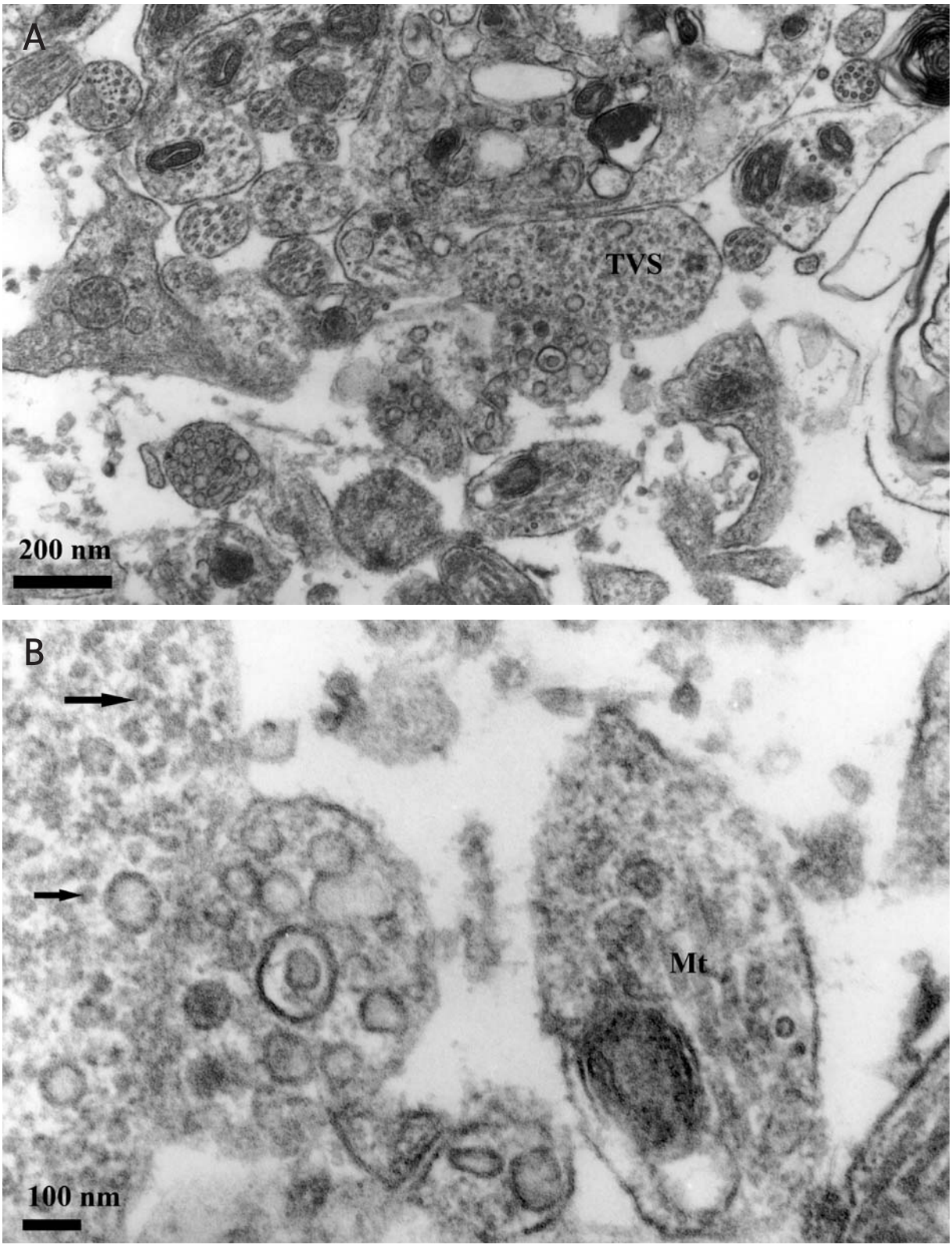

Fig. 5A-B. Low (A) and high (B) power electron microscopy picture to demonstrate a neuronal process containing TVS. A. TVS – tubulovesicular structures. B. Arrows (TVS), Mt – microtubules

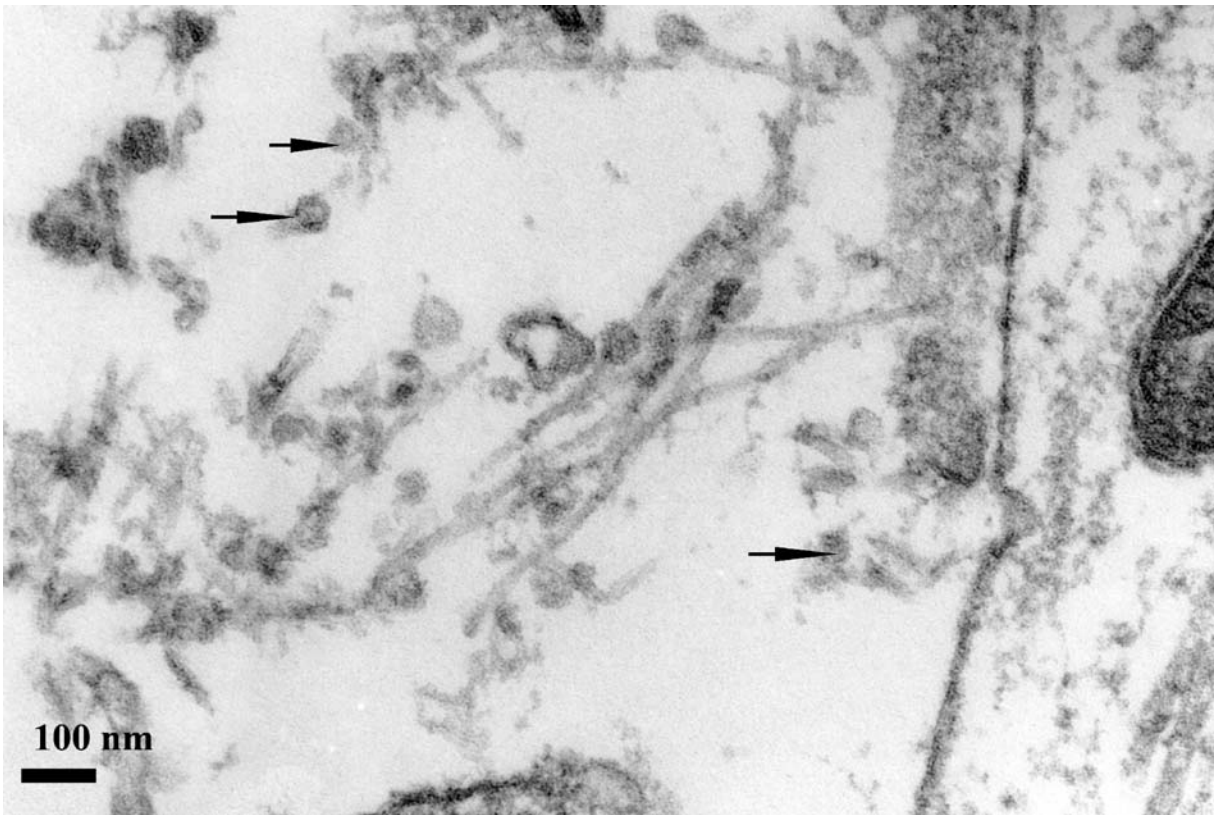

Fig. 6. TVS-like particles (arrows) attached to amyloid fibrils floating within loose plaques

Exactly how PrP^d exerts its deleterious effect on brain tissues is not clear. Many *in vitro* experiments suggest that PrP, or at least a part of its sequence, is intrinsically neurotoxic and that this neurotoxicity leads to apoptosis of affected neurons [7,37,44,46,60]. Whether the neurotoxic effect requires a β-pleated conformation of PrP^d is unknown. The same uncertainty applies to other "conformational disorders", such as Alzheimer's disease, in which Aβ peptide deposits extracellularly in a form of amyloid plaques. It is possible that amyloid plaques composed of fibrils in a β-pleated conformation are merely "tombs" of distorted protein which are too aggregated to be removed by naturally occurring cellular proteases.

The relation, if any, between "loose" plaques described here and the compact plaques (kuru and multicentric plaques) is completely uncertain. Two basic scenarios may be envisaged. The first is that the subependymal plaques precede the formation of compact plaques. This seems unlikely, however, because the location of compact plaques is different from that of loose plaques (compact plaques are encountered mostly in the cerebellum, "loose" plaques in the subependymal region). A more plausible hypothesis suggests that the electron-lucent environment in which amyloid plaques "float" makes them readily visible, which is not the case in the neuropil, which is, *per se*, highly fibrillar. To this end, when plaques are retrieved from formalin-fixed, paraffin-embedded material, most of the neuropil structure is destroyed and many PrP^d-amyloid fibrils become visible [43,47]. Thus, "loose" plaques are, in a sense, an artefact of the microenvironment that does not obscure their presence. It thus seems that simple PrP^d amyloid may take many different forms such as those discriminated by light microscopic studies.

The presence of tubulovesicular structures (TVS), 27-35 nm virus-like particles in close association with loose plaques, and also TVS-like particles attached to amyloid fibrils floating within loose plaques is intriguing. TVS have not only been found in all TSEs [42] but also were recently observed in scrapie-infected cells *in vitro* [48]. For those who are not totally convinced by the prion hypothesis, their close association with PrP^d amyloid fibrils may

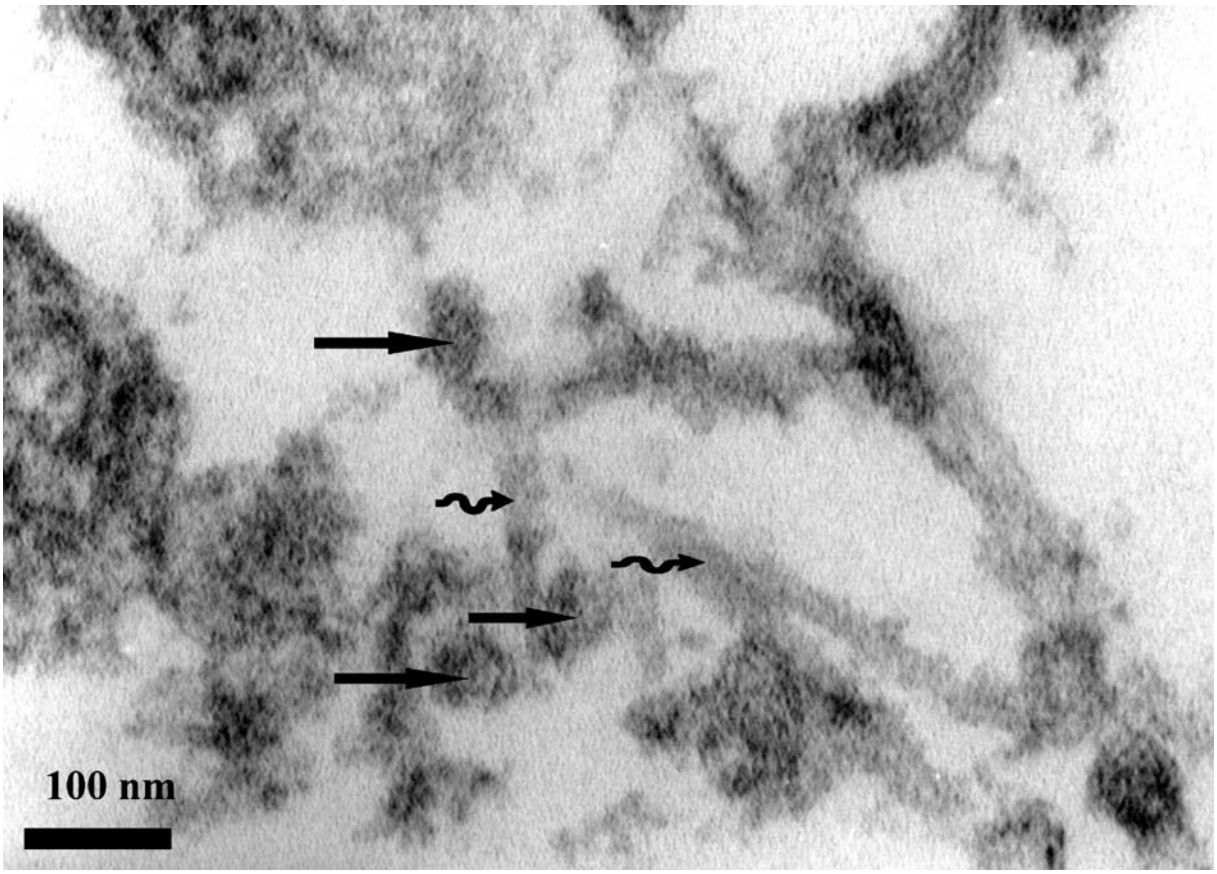

Fig. 7. High-power electron micrograph of TVS-like particles (arrows) attached to amyloid fibrils (spiral arrows) floating within loose plaques

readily explain the fact of an association of PrP^{d} and infectivity.

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