Original article

Folia Neuropathologica

Ultrastructural evidence of amyloid β-induced autophagy in PC12 cells

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Folia Neuropathol 2009; 47 (3): 252-258

Abstract

Herein we demonstrate that PC12 cells overexpress human amyloid β precursor protein bearing double Swedish mutation (AβPPsw), showing the phenotype characteristic for Alzheimer's disease (AD). Examination of cells at ultrastructural level revealed the intracellular presence of peptide aggregates. Furthermore, autophagy induction was found to be a hallmark of amyloid β-induced cytotoxicity. Importantly, autophagic vacuoles were co-localized within amyloid β (Aβ) deposits. This suggests the involvement of autophagy in amyloid β-elicited cell degeneration.

Key words: *amyloid β, Swedish double mutation, ultrastructure, PC12 cells.*

Introduction

Alzheimer's disease (AD) pathology is recognized by the formation of senile plaques and neurofibrillary tangles (NFTs). On one hand, the major component of senile plaques is the small amyloid β peptide (Aβ, that is a 39-43 amino acid fragment derived from targeted proteolysis of amyloid precursor protein (AβPP) by β- and γ-secretases [17]. On the other hand, NFTs are composed of hyperphosphorylated forms of the microtubule-associated protein tau [9]. It has been reported that γ-secretase complex contains a variety of proteins including presenilin-1 and presenilin-2. Mutations in various genes, such as AβPP and presenilin, are associated with early-onset familial Alzheimer's disease (FAD) evoked by increased production of Aβ oligomers and its deposition found in insoluble plaques [1,3,5,6,16]. A Swedish familial double mutation is located before the amyloid β peptide region of AβPP and results in the increased production and secretion of Aβ. Haass et al. [8] showed that in wild-type cells Aβ generation requires recurrent internalization and recycling of AβPP, whereas in the case of Swedish mutation the N-terminal β-secretase cleavage of Aβ occurs in Golgi-derived vesicles. Therefore, this cleavage is located in the same compartment as in α-secretase cleavage, which normally prevents Aβ production, explaining the increased Aβ formation by competition between α- and β-secretase.

Autophagy and its major type, macroautophagy, is the bulk protein degradation pathway associated with marked membrane dynamics. In response to various stimuli, such as starvation or humoral factors, an isolation membrane appears promptly in the cytosol,

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where it elongates to sequestrate cytoplasmic constituents. Subsequently, the edges of the membrane fuse together and form double-membrane structures termed autophagosomes [10]. Next, they mature into single membrane autophagolysosomes by fusing with late endosomes or lysosomes, at which time they acquire proteolytic enzymes [7].

Notably, ultrastructural analysis of biopsied brain specimens from patients suffering from Alzheimer's disease have revealed compartments of autophagic vacuoles which accumulate abnormally in affected neurons [18]. It has been suggested that autophagy may be up-regulated to eliminate abnormal intracellular proteins that would otherwise accumulate within cells as aggregates or inclusions [13]. Thus, suggested activation of autophagy within the cells over-expressing amyloid β (Aβ) protein seems reasonable. The aim of our study was to verify whether Aβ up-regulation in PC12sw-transfected cells could induce autophagy. Obtained results indicate that PC12sw cells could be a good experimental model to study the detailed molecular mechanisms of Aβ toxicity.

Materials and Methods

Cell culture

Human amyloid-β precursor protein (AβPP) bearing Swedish mutation-transfected (AβPPsw) stable cell clones were kindly provided by Professor Walter Müller from the Department of Pharmacology, Biocentre (University of Frankfurt, Germany). AβPPsw-transfected, as well as mock-transfected control PC12 cells were cultured in medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 5% horse serum (HS) and antibiotic mixture (50 U/mL penicillin, 50 µg/mL streptomycin) (Gibco Life Technologies, Paisley, United Kingdom). One day (24 h) prior to the experiment, confluent cells were then switched to post-mitotic status to induce quiescence (withdrawal from cell cycle) by replacing growth medium with 2% FCS/DMEM designated as a control medium (CTRL). In the above-mentioned conditions divisions of PC12 cells were completed.

Ultrastructural studies

Cells were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at 4°C. Cells were washed with the same buffer and post-fixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer for 1 h. Cells were dehydrated in a graded ethanol alcohol series, and embedded in Epon 812. Ultrathin sections were mounted on copper grids, air-dried, and stained for 10 min with 4.7% uranyl acetate and for 2 min with lead citrate. The sections were examined and photographed with a JEOL JEM 1011 electron microscope.

Post-embedment immunostaining

For immunocytochemical studies the cells were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M PBS (pH 7.4) for 2 h at 4°C. Next, cells were washed with the same buffer and post-fixed with 1% OsO_4 for 1 h. After dehydration cells were embedded in Epon 812 and ultrathin sections were processed according to the post-embedding procedure. The sections were mounted on Formvar-coated nickel grids, placed in 10% hydrogen peroxide for 10 min, rinsed in PBS for 30 min and further incubated with 5% BSA in PBS for 10 min. For single labelling rabbit polyclonal anti-MAP-LC3 IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or rabbit polyclonal anti-Aβ (1-40) (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) were diluted 1 : 20 in PBS. After 24 h at 4°C the grids were washed in PBS for 30 min and exposed to secondary anti-rabbit IgG conjugated with colloidal gold particles of 18 nm in diameter (Jackson Immunoresearch, West Grove, PA, USA) diluted 1 : 50 in PBS. After 1 h incubation in darkness at RT the grids were washed with PBS for 15 min, followed by distilled water for 15 min. Ultrathin sections were air-dried, and stained with 4.7% uranyl acetate for 10 min and with lead citrate for 2 min. The sections were examined and photographed with a JEOL JEM 1011 electron microscope.

Results

As has been previously shown, transfection of PC12 cells with AβPP bearing Swedish mutation (AβPPsw) results in a highly significant 4.8-fold increase in Aβ secretion to culture medium [4]. Our ultrastructural analysis revealed that AβPPsw overexpression also caused the formation of intracellular aggregates of Aβ (Fig. 1). In contrast, no such structures were detected in mock-transfected cells (Fig. 1A). Aβ deposits appear as a network of randomly orientated fibrous

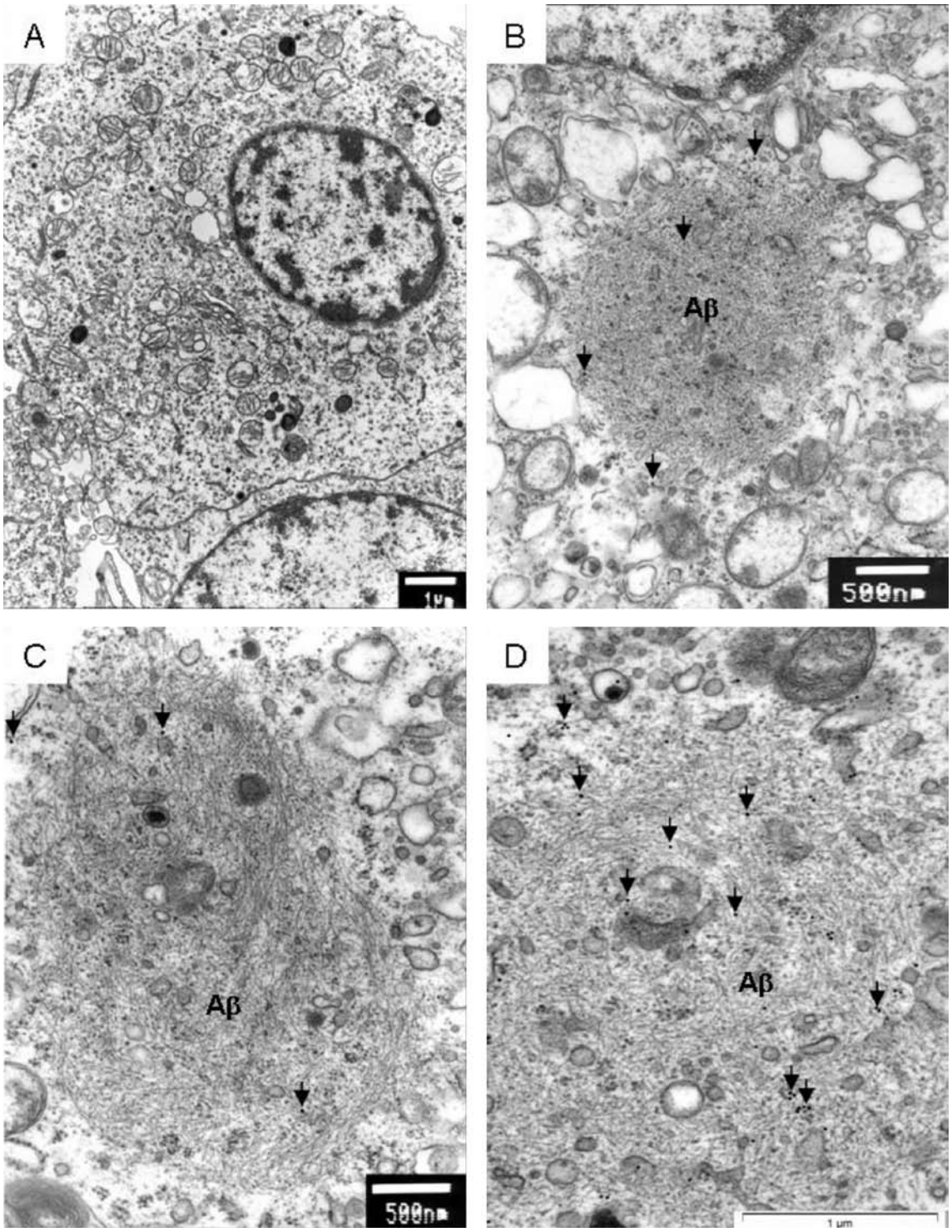

Fig. 1A-D. Ultrastructural morphology of control PC12 cells (A) or PC12 cells transfected with AβPPsw (B-D). Note the presence of protein deposits (Aβ) in cytoplasm of transfected PC12 cells, which were not found in control cells (A). Aβ deposits appear as a network of randomly orientated fibrous material localized in cell cytoplasm without limiting membrane. The presence of Aβ aggregates is positively confirmed by anti-Aβ IgG conjugated with 18 nm gold (arrows) staining (B-D).

material localized in cell cytoplasm without limiting membrane (Fig. 1B-D). To verify whether the accumulated protein is derived from AβPP protein proteolysis, immunocytochemical analysis was performed in order to detect the 40 amino acid fragment of Aβ. We found that gold particles were situated at Aβ cytoplasmic aggregates (Fig. 1D).

Evaluation of PC12sw cells demonstrated ultrastructural alterations with spectacular evidence of macroautophagy induction. As shown in Figure 2, numerous autophagic vacuoles (AV) were formed in PC12sw, but not in CTRL cells. Many of them met morphological criteria for autophagosomes (AU), including size > 0.5 µm in diameter, a double-limiting

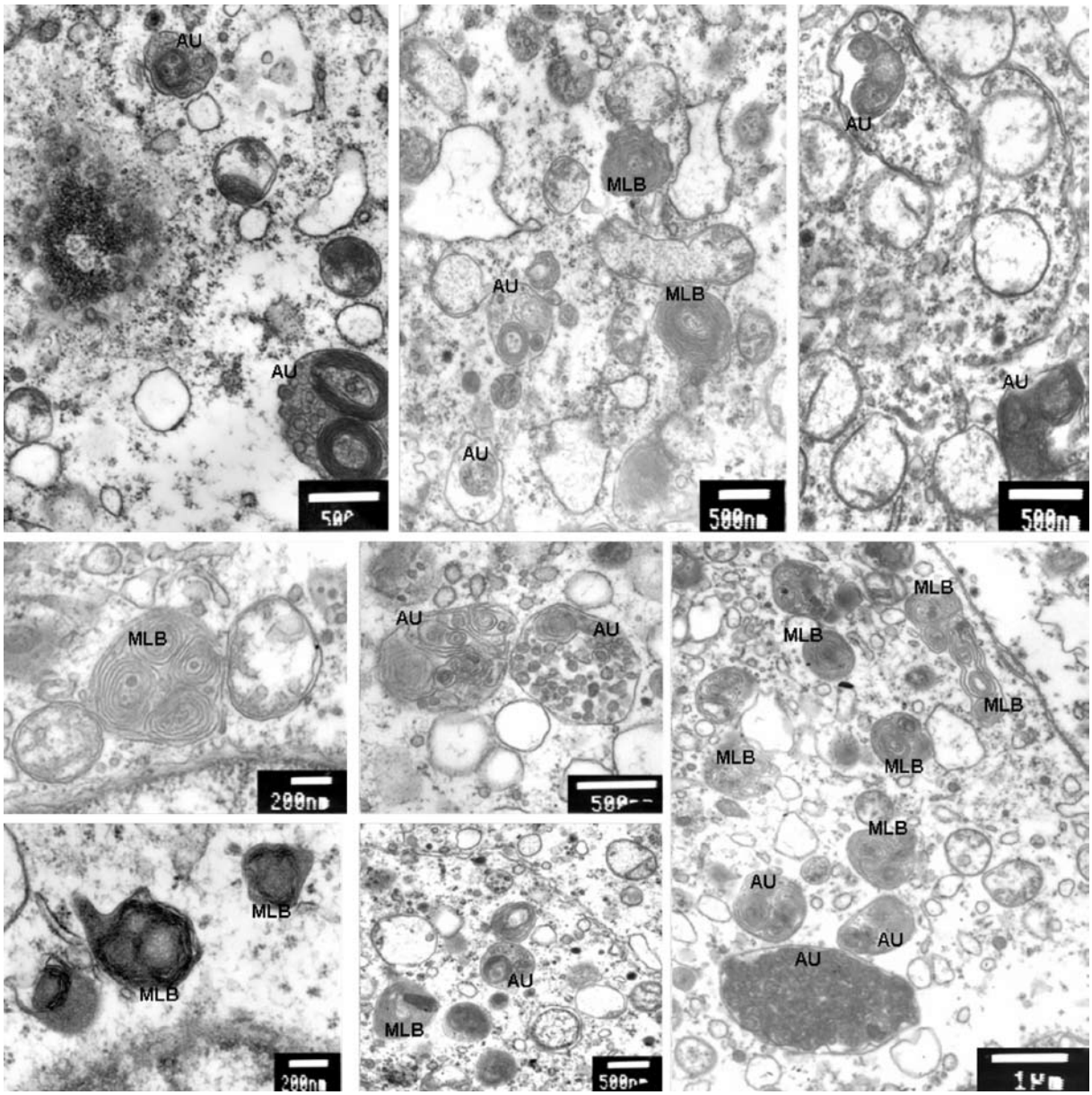

Fig. 2. Series of electron microscopy photographs showing autophagic vacuoles – autophagosomes (AU) – and multilamellar bodies (MLB) in AβPPsw-transfected PC12 cells. Autophagosomes are > 0.5 µm in diameter double-membrane vacuoles, sequestrating organelles (mitochondria, Golgi, endoplasmic reticulum). A second group of autophagic vacuoles is represented by dense multivesicular and multilamellar bodies (MLB), which are frequently distributed within the cytoplasm of PC12sw cells.

membrane, and the presence within a single vacuole of multiple membranous organelle-derived structures (mitochondria, Golgi, endoplasmic reticulum). A second group of autophagic vacuoles included dense multivesicular and multilamellar bodies (MLB), which were frequently distributed within the cytoplasm of PC12sw cells. Remarkably, in autophagic vacuoles Aβ aggregates were sequestrated together with above-mentioned cellular organelles (Fig. 3). Figure 3 demonstrates all stages of autophagic vacuole formation within Aβ deposits.

Finally, autophagy was confirmed by immunocytochemical analysis using antibody (Ab) that specifically distinguishes autophagosomes. This Ab is raised against microtubule-associated protein LC3 (MAP-LC3). In turn, LC3-II is the post-translationally modified product of the cytosolic microtubule-associated protein LC3-I. The induction of autophagy

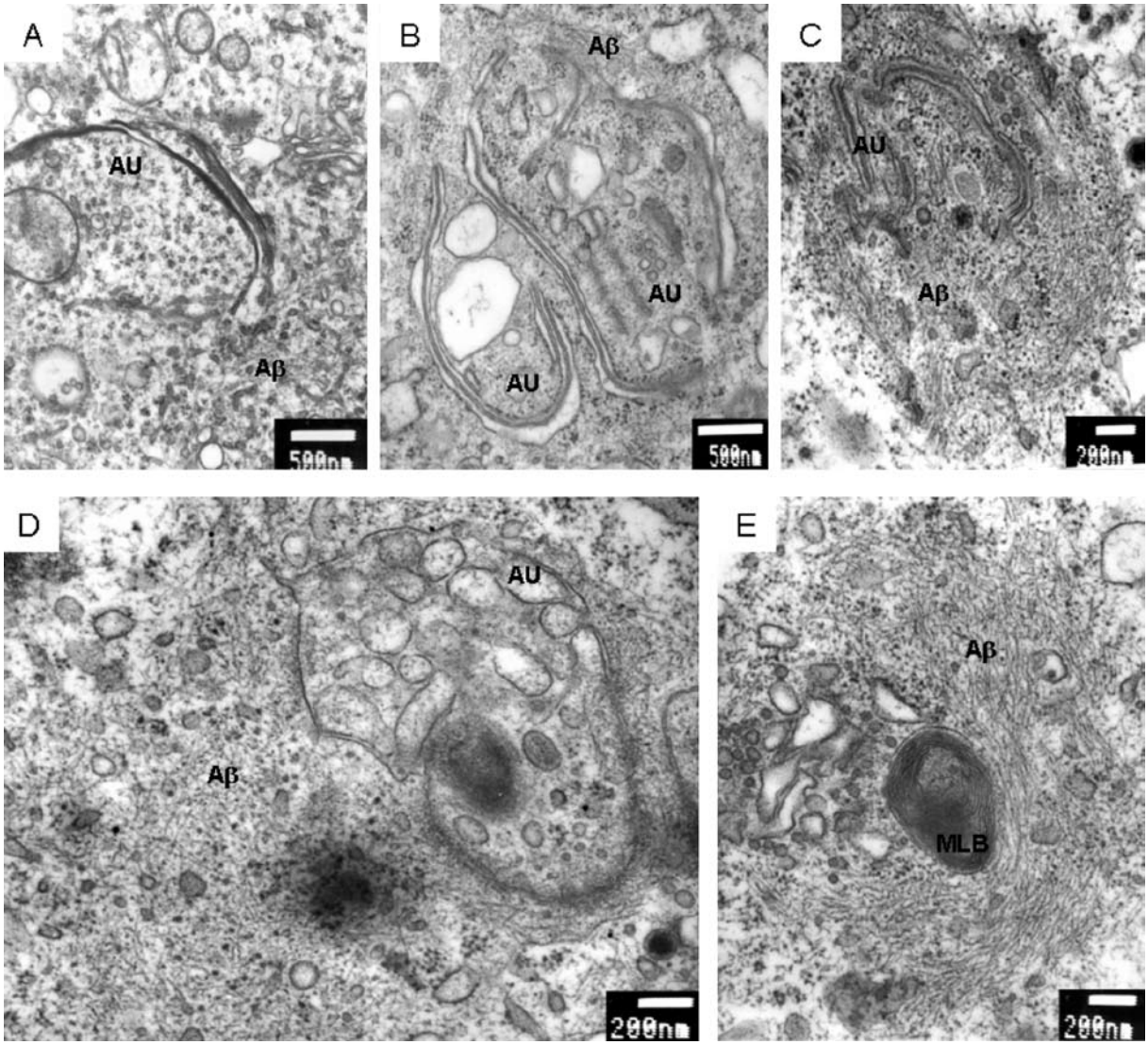

Fig. 3A-E. Sequence of electron microscopy photographs showing autophagic vacuoles co-localized with Aβ deposits in AβPPsw-transfected PC12 cells. The figure demonstrates all stages of autophagic vacuole formation within Aβ deposits: (A) the appearance of the isolation membrane within the cytoplasm; (B) elongation of the isolation membrane and sequestration of the organelles; (C) isolation membrane fusion; (D) double-membrane autophagosome formation; (E) the presence of MLBs as a result of mature, single-membrane autophagosomes and lysosomes or multivesicular bodies fusion.

promotes LC3-II formation and translocation to autophagosomes [2]. As shown in Figure 4, we did not find gold particles representing MAP-LC3-II on cellular compartments other than membranes of autophagosomes. These observations provide strong evidence for the relationship between Aβ deposits and autophagy in PC12sw cells. Most likely, Aβ deposits trigger autophagy, so more Aβ means more autophagic vacuoles in affected cells.

Discussion

This study demonstrates the consequences of excessive amyloid β peptide deposition in PC12 cells, stably transfected with human AβPP bearing "Swedish mutation". There are several papers showing marked biochemical changes in neurons during progression of Alzheimer's disease [4,11,15]. Considerable alterations in morphology of Alzheimer brains were also shown. We have previously reported that overexpression of AβPP in a PC12 cell line triggers cytological disturbances, which showed up as those typical for AD [12]. Our present *in vitro* experiment offers the attractive hypothesis that autophagy is subsequent to Aβ secretion in PC12sw cells. Consistently, the ultrastructural appearances of AβPP-transfected PC12 cells confirm the quantitative relationship between Aβ and autophagic vacuoles (AV) (Fig. 3). It is very probable that the formation of AV containing cytoplasmic aggregates of Aβ plays a significant protective role. This assumption is in agreement with Sahoo et al. [14], who found that the induction of autophagy by cysteine protease inhibitor results in inhibition of Aβ oligomerization and fibril formation in murine primary neurons. Moreover, Yu et al. [18] reported how autophagy is related to Aβ production. Their exhaustive electron and immunoelectron microscopic analyses revealed accumulation of LC3-

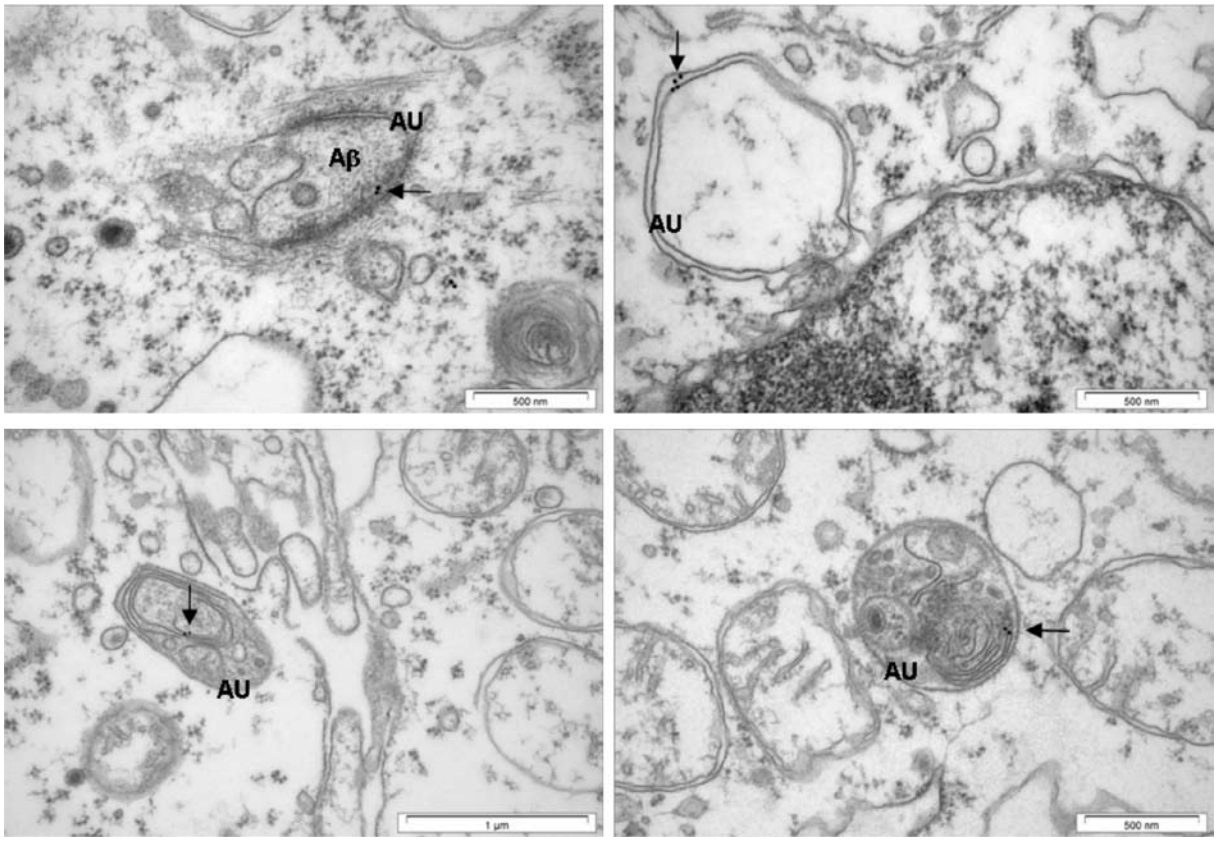

Fig. 4. Immunogold analysis of MAP-LC3 protein localization in PC12sw cells. Gold particles representing MAP-LC3 (arrows) are localized within autophagic vacuoles (AU). They were detected neither in control cells nor in other cellular organelles.

positive AV in brains of AD patients and in a mice model of AD as well as in the neural cell lines and in non-neuronal AβPP-expressing cells. Similarly to our results, the localization of presenilin 1, Aβ40, Aβ42 and nicastrin on AV membrane was detected. They found that autophagy was activated by Aβ production, and inversely, inhibited autophagy led to excessive Aβ production. Thus, further studies are required to verify whether autophagy in AβPP-transfected PC12 cells prevents Aβ-dependent cytotoxicity.

Acknowledgements

This work was supported by scientific network No. 28/E-32/BWSN-0053/2008 from the Ministry of Science and Higher Education.

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