Neuro-degenerative Diseases

Increased Aβ Production Leads to Intracellular Accumulation of Aβ in Flotillin-1-Positive Endosomes

Lawrence Rajendran[a]   Marlen Knobloch[c]   Kathrin D. Geiger[b]   Stephanie Dienel[a]
Roger Nitsch[c]   Kai Simons[a]   Uwe Konietzko[c]

[a]Max Planck Institute of Molecular Cell Biology and Genetics, and [b]Neuropathology, Uniklinikum,
Techical University of Dresden, Dresden, Germany; [c]Psychiatry Research, University of Zurich, Zurich, Switzerland

Abstract

Extracellular accumulation of Aβ in β-amyloid plaques is thought to be associated with the neurodegeneration observed in Alzheimer's disease (AD) patients, although a lack of correlation with cognitive decline raised doubts on this hypothesis. In different transgenic mouse models Aβ accumulates inside the cells and mice develop behavioral deficits well before visible extracellular β-amyloid accumulation. Here we show that intracellular Aβ accumulates in flotillin-1 positive endocytic vesicles. We also demonstrate that flotillin-1 is not only associated with intracellular Aβ in transgenic mice but also with extracellular β-amyloid plaques in AD patient brain sections.

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Introduction

The occurrence of intracellular Aβ accumulation has recently been shown by several groups in different transgenic mouse models and in postmortem tissue of Alzheimer's disease (AD) patients [1–5]. The nature of these accumulations, however, and whether they are toxic, needs further exploration. Our newly reported transgenic mouse model, the ArcAβ mouse that overexpresses human APP 695 with the Swedish and Arctic mutations in a single construct, showed consistent behavioral deficits before the deposition of β-amyloid plaques. These deficits correlated with a rise in intracellular punctate deposits of Aβ, most prominently in hippocampal, subicular and cortical regions [6]. To further elucidate the subcellular localization of these punctate Aβ deposits, we performed immunohistological double-staining with various organelle markers including flotillin-1 (reggie-2). Flotillin-1 belongs to the prohibitin (PHB) family of proteins that include flotillin-2, stomatins and others [7, 8]. Flotillins are lipid raft associated proteins [9] and are found at the plasma membrane and in endosomes and multivesicular bodies [10–12]. We provide evidence that intracellular Aβ accumulates in flotillin-1 positive vesi-

Uwe Konietzko
August Forel-Strasse 1
CH–8008 Zürich (Switzerland)
Tel. +41 44 634 8875, E-Mail uwekon@bli.unizh.ch

cles and that flotillin-1 is also localized in neuritic β-amyloid plaques of AD patient brains.

Materials and Methods

Transgenic Mice

In this study, ArcAβ mice previously described in Knobloch et al. [6] and their wt littermates were used. In brief, tg mice show about 6-fold overexpression of human APP695 containing the Swe (K670N + M671L) and Arc mutations (E693G) under the control of the prion-protein promoter, with constant levels of full-length APP and age-dependent increases in Aβ.

Immunostaining and Confocal Fluorescence Microscopy

Coverslip-grown Hela cells were fixed with 3.7% paraformaldehyde (PFA), washed with ammonium chloride, permeabilized with 0.1% Triton X-100 for 5 min, washed with PBS and blocked for 1 h with 0.2% BSA/0.2% Fish Skin Gelatin in PBS (blocking buffer). Cells were then incubated with primary antibodies (rabbit anti-flotillin-1, mouse anti-EEA-1 or mouse anti-giantin or mouse anti-Lamp) in blocking buffer for 1 h, subsequently washed thoroughly with PBS and the primary signal was detected with various fluorochrome (Cy3 or Cy5) conjugated anti-mouse or anti-rabbit antibodies. The images were acquired using Zeiss confocal microscope, LSM510.

Immunohistology

Mice were anesthetized (10 ml/g BW ketamine/xylaxine) and perfused transcardially with PBS. One hemisphere was fixed in 4% paraformaldehyde and embedded in paraffin. 5-μm sagittal sections were cut with a Leica RM 2135 microtome (Bannockburn, Ill., USA). Microwave pretreatment (10 min 85°C in citrate buffer) and 5 min submersions in 95% formic acid (FA) were done before immunostaining. After blocking of non-specific binding with 4% BSA, 5% goat serum and 5% horse serum at RT for 1 h, sections were incubated with both primary antibodies overnight at 4°C (6E10, Signet, 1:400; anti-Aβ40, Sigma, 1:200; Flotillin-1, BD Biosciences, Germany, 1:400). After washing with PBS, sections were incubated with fluorophore-conjugated antibodies for 2 h at room temperature (Cy5, 1:250, Cy2, 1:100, Jackson) and laser confocal microscopy was performed with a Leica TCS SP2.

Histology and Immunochemistry on Human Autopsy Tissues

The cases included 5 control brains (including 1 case with Parkinson's disease) and 6 cases with AD CERAD scores B–C). 5 μm thick sections of formalin-fixed and paraffin-embedded autopsy tissues of the left hippocampus and/or left parietal cortex of human brains were deparaffinized, stained with Gallyas silver stain [13] (reagents from Merck, Darmstadt, Germany) followed by immunochemistry for flotillin-1 or control conditions. The slides were first immersed in concentrated formic acid for 3 min, rinsed with PBS and then cooked in a vapor cooking apparatus (Multigourmet, Braun, Kronberg, Germany) for 20 min in 10 mM sodium citrate with 0.01% Tween, pH 6.5. The primary antibody was applied at a dilution of 1/100 overnight at 4°C. Development followed using the ABC indirect alkaline phosphatase kit rabbit IgG (Vector, Burlingame, Calif., USA) and Neu-Fuchsin (Sigma, St.Louis, Mo., USA) as a chromogen [14].

Results

Intracellular Punctate Deposits of Aβ in ArcAβ Mice Accumulate in Flotillin-1-Positive Vesicles

β-Amyloid plaque deposition and cerebral amyloid angiopathy are prominent features in ArcAβ mice between 9 and 15 months. Behavioral deficits, however, occur much earlier; these correlate with intracellular punctate Aβ deposits revealed by labeling with antibodies directed against the Aβ domain and the failure of APP C-terminal antibodies to stain these structures [6]. These intracellular Aβ deposits are visible from 3 months onward in the hippocampus, subiculum and cortex but are undetectable in the cerebellum and brainstem, the latter being brain regions unaffected in AD.

Double-staining of brain sections of 7-month-old ArcAβ mice showed intracellular punctate Aβ (6E10, blue) that were frequently surrounded by flotillin-1 (green)-positive structures (fig. 1). The flotillin staining did not fully encircle the Aβ deposits but rather concentrated in several patches at the edge of the Aβ deposits. The 6E10 antibody also detects sAPPα; therefore, we additionally stained with an antibody specific for $A\beta_{40}$ (insert in upper panel of fig. 1), clearly identifying intracellular deposits as Aβ.

Flotillin-1 Is Associated with Early and Late Endosomes in the Endocytic Pathway

To further analyze the nature of the flotillin-positive vesicles, we characterized these vesicles in a cell culture system. We used HeLa cells to colocalize endogenous flotillin with various subcellular markers (fig. 2). Partial overlap of flotillin was seen with the early endosomal marker, early endosomal antigen-1 (EEA-1) and a significant overlap with Lamp-1, a marker for late endosomes and lysosomes. However, no significant colocalization was observed with giantin, a Golgi marker. Colocalization of flotillin with several organelle-specific rab-GTPases showed that flotillin predominantly labeled the late endosomes (data not shown), consistent with other reports [11]. We and others have previously shown that Aβ peptides are found in multivesicular bodies (MVBs) and fusion of MVBs with the plasma membrane releases a minor amount of Aβ in association with exosomes [15, 16]. Together these data suggest that intracellular Aβ resides in late endosomes/MVBs.
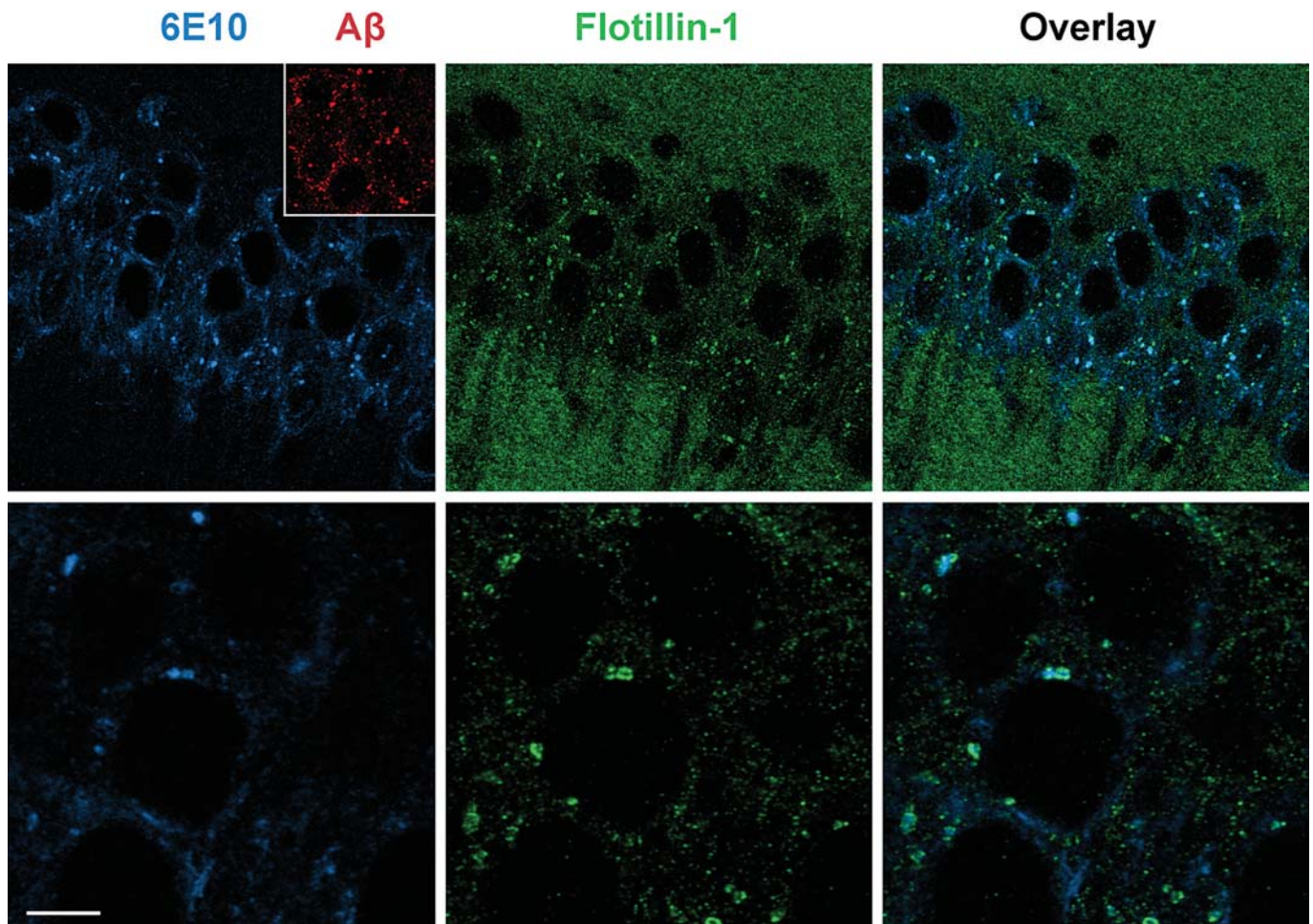
Fig. 1. Flotillin-1 patches surround intracellular Aβ aggregates. Brain sections of ArcAβ transgenic mice were stained for Aβ with the 6E10 antibody (blue) and flotillin-1 (green). Upper panel shows a section of the hippocampal CA1 pyramidal soma layer. Enlargement (lower panel) shows patchy flotillin-1 staining surrounding intracellular punctate Aβ deposits. Insert in upper panel (red) shows staining of intracellular punctae by an antibody specific for $A\beta_{40}$. Scale bar = 20 μm in the upper and 7 μm in the lower panel.

Flotillin-1 Is Enriched in Neuritic Plaques of AD Patients

As a result of MVB fusion with the plasma membrane, the exosomes that contain Aβ together with flotillin are released and might participate in β-amyloid plaque formation. We analyzed the distribution of flotillin-1 in brain sections of AD and Parkinson's disease patients and control subjects as described [16]. Most AD cases showed swollen, vacuolated, or tangle-bearing neurons with strong flotillin-1-expression (fig. 3a). There was immunoreaction (IR) in the processes of single astrocytes within the gray matter of areas showing degenerative pathology (fig. 3b). The flotillin-1-positive astrocytes consisted mainly of cells with a nucleus slightly increased in size and with less chromatin density, pointing towards a partial activation. Diffuse β-amyloid plaques showed mostly no increase of flotillin-IR over the matrix background. However, neuritic tangle-bearing plaques often showed increased flotillin-1-IR organized along the fibrillary component of the plaques (fig. 3c).

In two of the normal brains flotillin-1-IR was found in single astrocytes of the gray matter (fig. 3d). The others showed only slight diffuse staining of the extracellular matrix. Flotillin-1 was usually not detected in neurons, oligodendroglia and microglia of normal controls. The
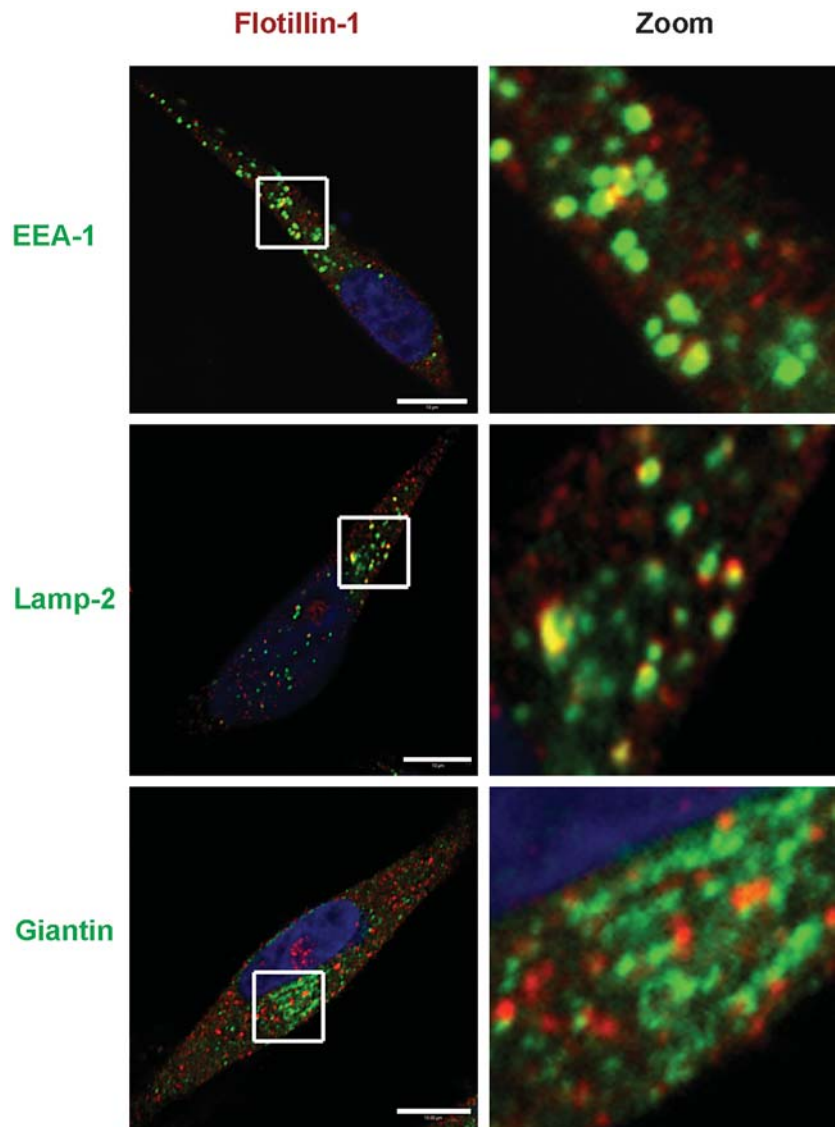
Fig. 2. Colocalization of flotillin-1 with endosomal but not with Golgi markers. HeLa cells were grown on coverslips and immunostained for endogenous flotillin-1 (red) and endosomal markers EEA-1 (green, upper panel) and Lamp-1 (green, middle panel) and Golgi marker Giantin (green, lower panel). Scale bar = 10 μm.

case with Parkinson's disease did not differ from the normal control cases (fig. 3e). Negative reaction controls showed no flotillin-1-IR (fig. 3f). The cases with AD showed two distinct partially overlapping patterns of flotillin-1-IR. There was expression of the protein in pathologically altered neurons and in association with neurofibrillary tangles. Diffuse β-amyloid plaques without neurofibrils showed only minimal expression of flotillin-1. By morphological criteria expression of flotillin-1 in astrocyte processes appeared to be associated with astrocyte activation. These results show that flotillin-1 is present in extracellular β-amyloid plaques and in tangle-bearing neurons.

Discussion

Our present study shows that intracellular Aβ accumulates in a subset of endocytic vesicles that are positive for the lipid raft-associated protein, flotillin-1. In ArcAβ mice, which exhibit consistent behavioral deficits before the deposition of β-amyloid plaques, these Aβ-laden endocytic vesicles might be involved in the mechanisms causing the cognitive impairment. We and others previously reported that generation of Aβ occurs in early endosomes after endocytosis of APP [16–19] where β- and γ-cleavage occurs [20]. We also showed that Aβ produced in early endosomes is retrogradely transported to late en-
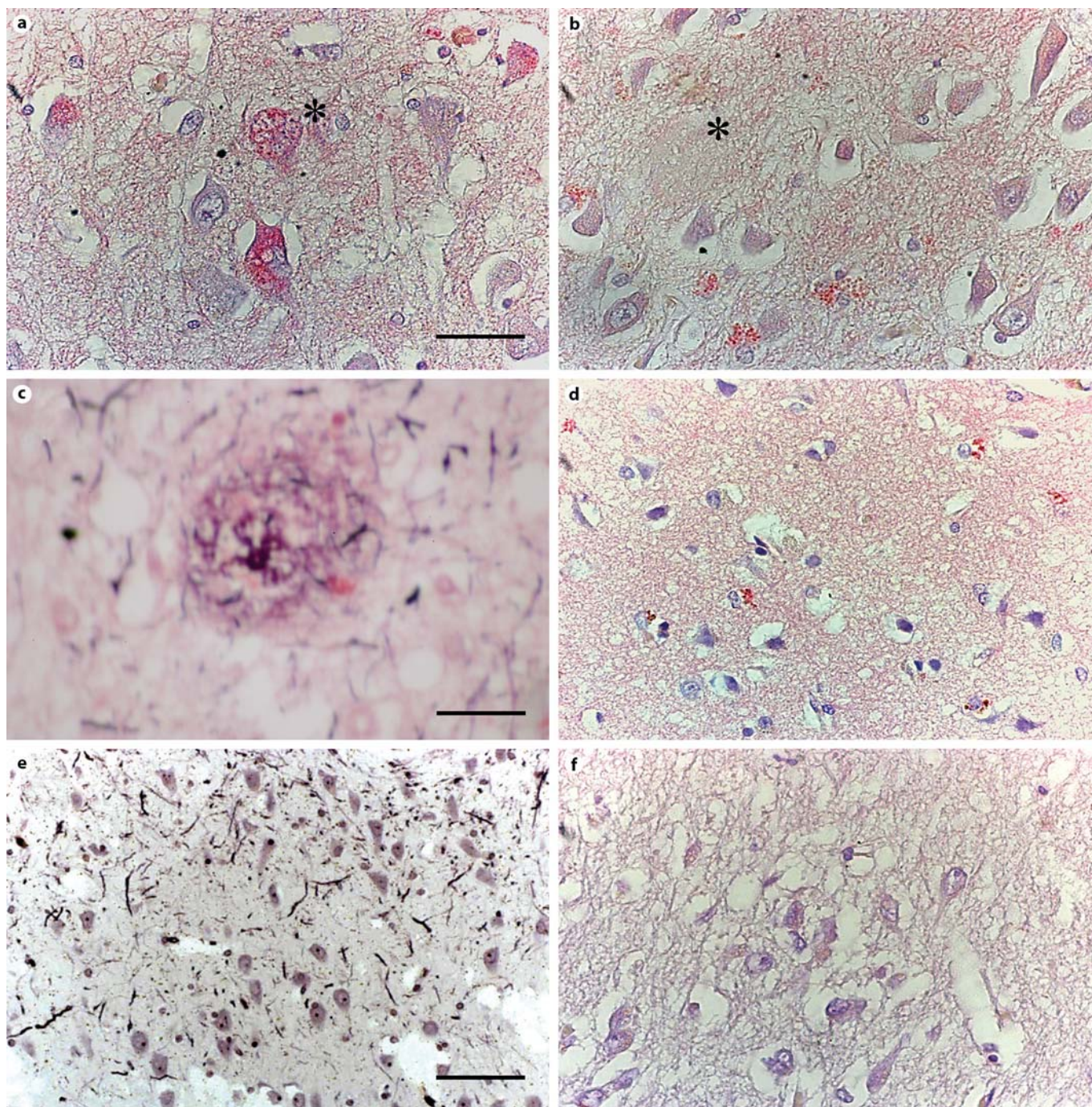
Fig. 3. Flotillin-1 in human autopsy tissues from patients with Alzheimer's disease. Flotillin-1 immunocytochemistry was combined with Gallyas staining for neurofibrillary tangles in **c** and **e**. **a** Flotillin-1 in tangle-bearing, pathologically altered swollen neurons, but not in intact neurons (*) of a patient with AD. The comparatively low abundance of neurofibrils in this patient appears noteworthy (AD CERAD age-related plaque score, **b**). Scale bar = 40 μm. **b** Different area (parietal cortex of the same patient as in **a** with flotillin-positive astrocytes, which are occasionally seen in normal brain as well (**d**) and a mostly flotillin-1-negative diffuse β-amyloid plaque (*). Scale bar = 40 μm. **c** Neuritic plaque in a different patient with AD bearing a high number of neurofibrils. (AD CERAD age-related plaque score, **c**). In this case, flotillin-1 is organized along neurofibrillary tangles and partially associated with β-amyloid deposits. Scale bar = 25 μm. **d** Normal brain with single, flotillin-1-positive (red) astrocyte processes in the grey matter of the parietal cortex. Scale bar = 40 μm. **e** Parkinson's disease brain. Flotillin-staining combined with Gallyas stain. Scale bar = 80 μm. **f** Negative control with omission of the primary antibody. Scale bar = 40 μm.

dosomes/MVBs [16]. As a result of the fusion of MVBs with the plasma membrane, the intraluminal vesicles of MVBs are released into extracellular space as exosomes. We hypothesized that the Aβ associated with exosomes could act as a nucleation factor for amyloid plaque formation. Our current work shows that preceding the detection of extracellular plaque formation, Aβ accumulates intracellularly in punctate structures that are frequently surrounded by flotillin-1 immunoreactivity. We conclude that the intracellular Aβ structures detected in ArcAβ mice represent membrane-delimited endosomal compartments. The fact that we did not observe constant increases in intracellular Aβ with increasing age of the animals would then implicate that intracellularly generated Aβ does not irreversibly accumulate in neurons. Aβ could be removed by degradative pathways (autophagosomes) [21] or even exocytosed via exosomes [16] into the extracellular space, affecting synaptic transmission [22, 23]. What is the significance of intracellular Aβ trapped in these endosomal structures? MVBs have been shown to present an optimal environment for fibrillation of Pmel1, an amyloid-forming protein that is involved in melanosome biogenesis and melanin polymerization [24]. Though our current study has not provided any evidence for Aβ oligomers/fibrils in these vesicles, it is tempting to propose that a similar mechanism could exist whereby intracellular Aβ oligomerizes already in MVBs. Flotillin-1 could stabilize these compartments or could also act as a raft scaffolding protein and thereby promote amyloidogenic cleavage in raft domains [19]. Furthermore, flotillin-1 has been shown to interact directly with APP [25] and several groups reported the presence of flotillin-1 in β-amyloid plaques [26–28], supported by our current findings.

To summarize, Aβ is generated in early endosomes and is retrogradely transported to the flotillin-1 containing late endosomes/MVBs. Aβ may undergo oligomerization in these raft-enriched structures, stabilized by flotillin, and could be subsequently released from the cells via exosomes, affecting synapse function. Furthermore, these exosome-associated Aβ could form a nucleation center for β-amyloid plaque formation.

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