

Intraneuronal Alzheimer A β 42 Accumulates in Multivesicular Bodies and Is Associated with Synaptic Pathology

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A central question in Alzheimer's disease concerns the mechanism by which β -amyloid contributes to neuropathology, and in particular whether intracellular versus extracellular β -amyloid plays a critical role. Alzheimer transgenic mouse studies demonstrate brain dysfunction, as β -amyloid levels rise, months before the appearance of β -amyloid plaques. We have now used immunoelectron microscopy to determine the subcellular site of neuronal β -amyloid in normal and Alzheimer brains, and in brains from Alzheimer transgenic mice. We report that β -amyloid 42 localized predominantly to multivesicular bodies of neurons in normal mouse, rat, and human brain. In transgenic mice and human Alzheimer brain, intraneuronal β -amyloid 42 increased with aging and β -amyloid 42 accumulated in multivesicular bodies within presynaptic and especially postsynaptic compartments. This accumulation was associated with abnormal synaptic morphology, before β -amyloid plaque pathology, suggesting that intracellular accumulation of β -amyloid plays a crucial role in Alzheimer's disease. (*Am J Pathol* 2002, 161:1869–1879)

β -amyloid (A β) plays a central role in the pathogenesis of Alzheimer's disease (AD).¹ Increasing reports suggest that the A β 42 form of A β can accumulate within neurons with aging and that intraneuronal A β may be directly involved in the pathogenesis of AD.^{2–4} AD transgenic mouse studies indicate that synaptic, behavioral, and physiological functions begin to decline^{5–7} as brain A β levels rise^{8,9} before the appearance of A β plaques. These studies suggest that intracellular A β accumulation may be important in AD.

Although A β 40 is the predominantly secreted A β species in tissue culture, the longer A β 42 is more important in AD. A β 42 is increased in familial AD (FAD) because of mutations in the amyloid precursor protein (APP) or presenilins 1 and 2, and it accumulates as the first A β species in Down's syndrome and AD.¹ Cell biological studies indicate that A β generation occurs in the Golgi apparatus (Golgi), especially the *trans*-Golgi network, and to a lesser extent in the endoplasmic reticulum (ER). The ER appears to be more selective for A β 42 than A β 40 generation.^{3,4} Moreover, the ER pool of A β 42 appears to be secreted less efficiently than that from the Golgi. A β peptide generation also occurs in the endocytic pathway after internalization from the plasma membrane.^{10,11}

AD pathogenesis has traditionally been characterized by extracellular, aggregated, and plaque-associated A β . However, the extent to which extracellular A β causes cell death in AD is unclear. An increasing number of reports demonstrate behavioral, synaptic, physiological, and oxidative abnormalities months before the appearance of A β plaques in FAD transgenic mice that develop AD-like plaque pathology.^{5,6,9,12} In Tg2576 mice, which express the human APP Swedish 670/671 FAD mutation,¹³ brain levels of A β rise sharply as measured by enzyme-linked immunosorbent assay (ELISA) at 6 to 10 months,⁹ before development of plaques, which appear at ~11 to 13 months.¹⁴ Preplaque increases in soluble A β 42, but not A β 40, have been reported in Down's syndrome brain.¹⁵ Studies of postmortem human brain show that soluble A β correlates better than insoluble A β or extracellular A β plaques with cognitive dysfunction in patients with AD.¹⁶ Thus, soluble A β 42 protofibrils may play a critical pathogenic role.¹

The location of the soluble A β 42 protofibrils and how they cause toxicity are of growing interest. A β protofibrils are preferentially generated within cells rather than extracellularly.¹⁷ Neuronal NT2 cells contain abundant intracellular A β 42, which increases dramatically with time

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(aging) in culture.¹⁸ Transgenic mice expressing a FAD mutant presenilin 1 were reported to develop intraneuronal A β 42 accumulation and neurodegeneration.¹⁹ Intracellular A β accumulation has been associated with pathology in inclusion body myositis²⁰ and mucopolysaccharidosis.²¹ Thus, cumulative indirect evidence suggests that there may be a pathological role for neuronal A β accumulation in AD. Using three different well-characterized pairs of polyclonal antibodies against A β 40 or A β 42, we previously reported that intraneuronal A β 42, as viewed by light microscopy, accumulates especially within AD-susceptible pyramidal neurons in postmortem tissue of patients with clinically defined early cognitive impairment.² Intraneuronal A β 42, but not A β 40, accumulation with AD pathology now has been reported by light microscopy by several other groups.^{22–26} In the present study, the subcellular localization of A β within neurons was examined using immunogold electron microscopy (EM) to localize where brain A β increases occur before the development of plaques, in a mouse model of AD-like A β pathology. We provide evidence implicating intraneuronal A β 42 accumulation in synaptic pathology both in AD transgenic mouse and human AD brains.

Materials and Methods

Antibodies

Monoclonal antibodies to the C-terminus of A β 40 and A β 42 were MBC40 and MBC42, respectively, generated by Dr. Haruyasu Yamaguchi, Gunma University, Gunma, Japan. MBC42 does not recognize A β 40 or A β 43.²⁷ The specificities of MBC40 and MBC42 antibodies were determined by immunoprecipitation of human APP-transfected neuroblastoma (N2a) cells, synthetic A β 40 or A β 42 with MBC40 or MBC42 antibody, followed by Western blotting with 6E10 monoclonal antibody (Signet Laboratories, Dedham, MA) as previously described.²⁸ A β 42 immuno-EM results were confirmed using a commercially available polyclonal anti-A β 42 antibody (Chemicon Inc., Temecula, CA). The C-terminus of full-length APP was recognized using antibody 369.²⁹

Animals

Well-established Tg2576 mice ($n = 22$) with the human APP Swedish 670/671 mutation of varying ages (four, 2 months; three, 3 months; two, 4 months; one, 8 months; three, 9 months; four, 10 months; two, 11 to 12 months; two, 16 to 17 months; one, 20 months) were used in this study. APP knockout mice and wild-type littermates ($n = 4$, each; two 6-month-old and two 12-month-old mice, each) were generously provided by Dr. Hui Zheng, Baylor College of Medicine, Houston, TX. Sprague-Dawley rats (300 to 325 g) were obtained from Taconic Farms (Germantown, NY). All methods were approved by the Weill Medical College of Cornell University Institutional Animal Care and Use Committee and conformed to National Institute of Health guidelines.

Human Brain Tissue

Human cortical brain tissue was obtained from the Department of Pathology, Weill Medical College of Cornell University as a result of neurosurgical procedures unrelated to this study. Neurosurgical AD cortical tissue was obtained ($n = 2$, ages 54 and 62 years). Relatively normal cortical brain tissue was obtained from emergency aneurysm clipping and benign tumor resection ($n = 2$, ages 44 and 54 years).

Pre-Embedding Immunogold Electron Microscopy

Mice and rats were anesthetized with sodium pentobarbital (150 mg/kg, i.p.) and perfused via the ascending aorta with 3.75% acrolein (Polyscience, Warrington, PA) and 2% paraformaldehyde in 0.1 mol/L of phosphate buffer (pH 7.4). Human brain biopsy tissue was rapidly immersion-fixed with 1.875% acrolein and 2% paraformaldehyde in 0.1 mol/L of phosphate buffer. Brain tissue was cut (40- μ m thick) on a vibrating microtome and treated with 1% sodium borohydride as previously described.³⁰

Some sections were processed for EM using immunoperoxidase, as described previously,³⁰ that revealed an analogous staining pattern (predominantly MVBs) with MBC42 antibody, but the subcellular localization was not as discreet as with immunogold. For immunogold labeling, free-floating sections were labeled with either MBC42, MBC40, 369, or Chemicon A β 42 antibodies by the immunogold-silver procedure of Chan and colleagues.³¹ For the processing, the tissue was incubated in goat anti-mouse (for MBC40 and MBC42) or goat anti-rabbit (for 369 and Chemicon anti-A β 42 antibody) IgG conjugated to 1-nm gold particles (Amersham, Arlington Heights, IL) in 0.01% gelatin and 0.08% bovine serum albumin in phosphate-buffered saline (PBS). The conjugated gold particles were enhanced by treatment with silver solution (IntenSE, Amersham).

Dual immuno-EM localization was performed as previously described³⁰ now using polyclonal rabbit anti-APP (369) and monoclonal anti-A β 42 (MBC42). Sections were incubated with both 369 and MBC42, and then processed first for immunoperoxidase localization of APP and then with the immunogold-silver method for localization of A β 42. A β 42 antibody concentrations were 1:50, A β 40 antibody concentrations were 1:20, and APP antibody concentrations were 1:500. Sections were fixed in 2% osmium tetroxide in phosphate buffer, embedded in EMBed 812, sectioned (65- to 76-nm thick) and counterstained with uranyl acetate and Reynolds' lead citrate.³⁰ Final preparations were examined with a Philips CM10 electron microscope. Morphological terminology is consistent with Peters and colleagues.³² Quantification was done by counting gold particles from random images taken at the same magnification from analogous cerebral cortical regions with neuronal soma (layer 5) or processes of either 2- or 10-month-old Tg2576 mice. Student's *t*-test was used for statistical analyses. Final illus-

trations were generated from scanned photographic prints (for Figure 1E; Figure 3, B and C; Figure 4A; and Figure 5, A, B, and C) or from a high-resolution digital imaging charge-coupled device camera system (Advanced Microscopy Techniques Corp., Danvers, MA) and processed on a Power Macintosh 8500/120 using Adobe Photoshop 6.0 (Adobe System, Mountain View, CA) and Quark X-Press 3.32 (Quark, Denver, CO).

Immunoprecipitation/Western Blot for A β

Equal amounts (25 mg) of brain tissue snap-frozen in liquid nitrogen derived from analogous cortical regions from brains of Tg2576 mice sacrificed at different ages (3, 9, and 16 months) or N2a cells stably transfected with Swedish 670/671 FAD mutation human APP (generously provided by Drs. Gopal Thinakaran and Sangram Sisodia, University of Chicago, Chicago, IL) were assayed for determination of A β levels as previously described for cell lysates²⁸ with slight modifications. Samples from frontal and parietal cortex were analyzed for A β . Brain samples or N2a cells were lysed in 6% or 4% sodium dodecyl sulfate, respectively, vortexed, and heated at 95°C for several minutes, followed by sonication. Samples were spun at 15,000 \times g, and supernatants adjusted to 190 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 8.3, 6 mmol/L ethylenediaminetetraacetic acid, and 2.5% Triton X-100 and incubated overnight with antibody 4G8 (Signet Laboratories), followed by addition of secondary rabbit anti-mouse antibody (Cappel) for 1 hour and then addition of protein A-Sepharose beads (Pharmacia) for 2 hours (all at 4°C). Samples were run on 10 to 20% Tris-Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels followed by electrophoretic transfer to polyvinylidene-difluoride membrane (Millipore, Bedford, MA). Polyvinylidene-difluoride membranes were boiled in PBS for 5 minutes, followed by Western blotting with 6E10 for human A β and visualization after enhanced chemiluminescence on Kodak X-OMAT AR5 film (Eastman-Kodak, Rochester, NY).

Results

Intraneuronal A β 42 Resides on MVBs

In agreement with a previous report,²⁷ standard fixation of normal rat brain for EM with paraformaldehyde/glutaraldehyde revealed primarily isolated A β 42 immunolabeling at the plasma membrane (data not shown). In contrast, fixation with a mixture of acrolein and paraformaldehyde used in the present study, which allows for more optimal antigen detection by immuno-EM,³³ revealed marked intraneuronal A β , especially A β 42. In normal mouse (Figure 1A) and rat (Figure 1B) brain, A β 42 was most prominent on multivesicular bodies (MVBs), especially of pyramidal neurons in the cerebral cortex and hippocampus, which are particularly prone to AD neuropathology. Intraneuronal A β 42 was less pronounced in neurons known to be relatively resistant to AD, such as in the basal ganglia. More than half of A β 42 gold particles labeling identifiable organelles within neu-

rons in the brain of normal mouse or rat were found on MVBs. Moreover, rapidly fixed brain tissue from patients undergoing brain surgery for noninvasive neurological disease ($n = 2$, ages 44 and 54) also revealed A β 42 especially on MVBs within neurons (Figure 1C). In contrast to A β 42, neuronal A β 40 was not convincingly evident in normal mouse, rat, or human brain.

The monoclonal anti-A β 42 antibody²⁷ did not show cross-reactivity to full-length APP or APP C-terminal fragments (CTFs) by Western analysis of either APP-transfected neuroblastoma (N2a) cells or Tg2576 mouse brain (Figure 1D). Peptide competition abolished A β 42 detection both by Western blot and by immunohistochemistry. Lack of cross-reactivity with APP also can be deduced from the fact that full-length APP/CTFs localized predominantly to the Golgi, although a minor amount was also detected in MVBs, of neuronal cell preparations (using antibody 369 directed at the C-terminus of APP).³⁴ Golgi predominance of full-length APP staining by immuno-EM with antibody 369 was now observed within neurons of the brain, and immuno-EM double labeling was used to demonstrate the differential staining of full-length APP/CTFs and A β 42 (Figure 1E). We confirmed our MBC42 A β 42 immuno-EM labeling with the widely used polyclonal A β 42 antibody from Chemicon, which gave analogous A β 42 immunoreactivity (data not shown). In addition to the predominant site of A β 42 on the outer membrane of small MVBs (~60% of identifiable labeling in young 2- to 3-month-old mouse neuronal soma), A β 42 immunogold particles were observed on very small vesicles (~35% of identifiable labeling). Furthermore, immunogold labeling also was observed on ER (~5% of identifiable labeling; Figure 1A), similar to that previously observed in cultured neurons infected transiently with Semliki Forest virus containing human APP.³⁵ A β 42 was only occasionally seen at the plasma membrane, and in contrast to full-length APP, only a minor amount of A β 42 was observed on the Golgi apparatus compared with its predominance on MVBs (Figure 1).

APP Knockout Mice Lack Intraneuronal MVB A β 42

To further confirm the specificity of the A β immunoreactivity, well-characterized APP knockout mice ($n = 4$)³⁶ were compared with wild-type mice ($n = 4$) of equal age (two 6-month-old and two 12-month-old littermates) that were perfused and processed for immunogold EM in parallel. APP knockout mice did not demonstrate the A β 42 on MVBs observed in normal wild-type mice (compare Figure 2, A and B).

Intraneuronal A β 42 Increases with Aging

A β was examined by immuno-EM in Tg2576 transgenic mice. Two independent previous A β ELISA studies demonstrated exponential brain A β increases occurring before A β plaque deposition in Tg2576 mice.^{8,9} We used A β immunoprecipitation and Western blot to further confirm brain A β increases in our Tg2576 mice and observed

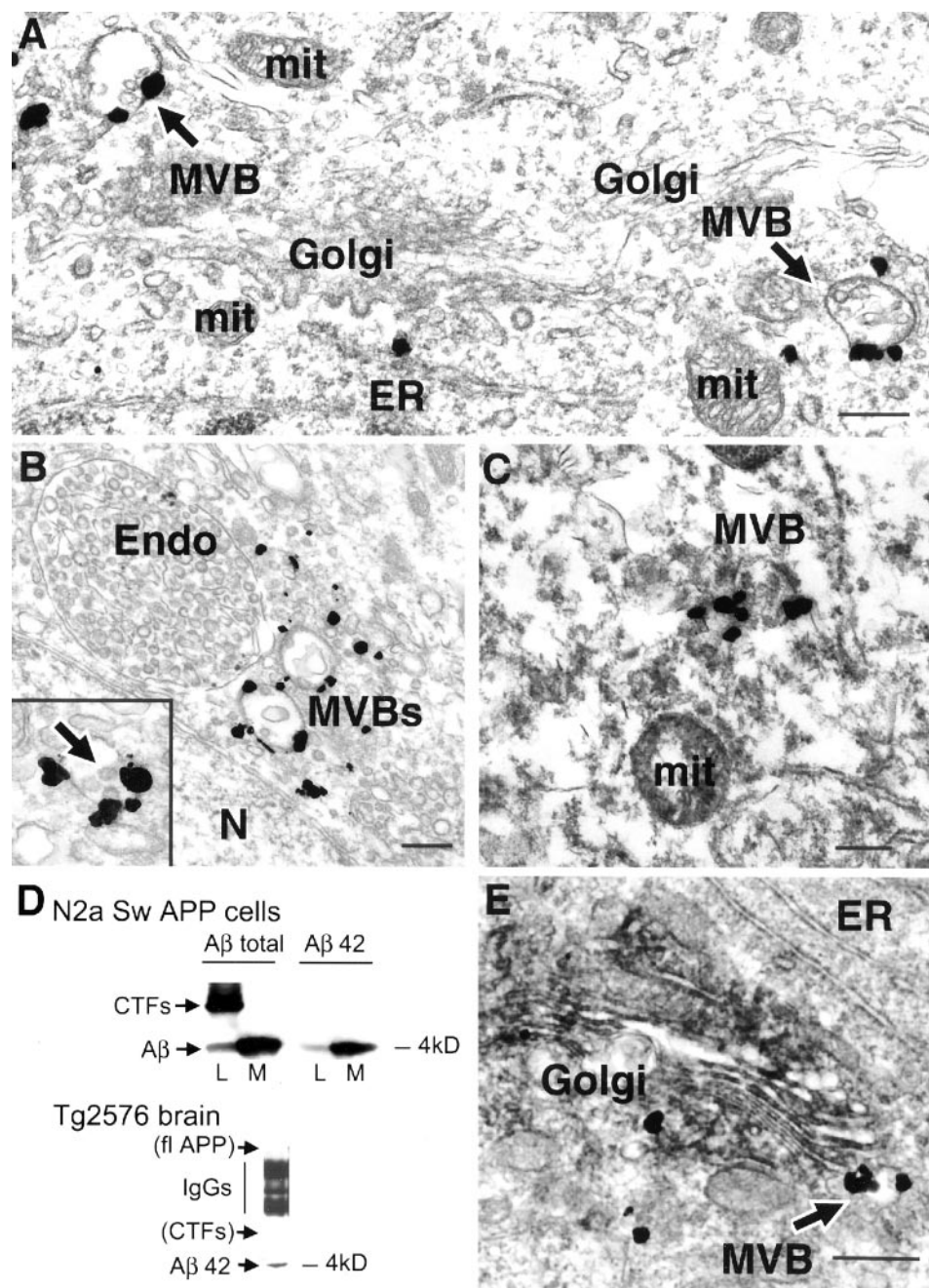


Figure 1. Ultrastructural localization of Aβ42 in neurons of normal mouse, rat, and human brain. **A:** Prominent Aβ42 immunogold labeling in MVBs (arrows) within a neuronal perikaryon in hippocampus (CA1) of a normal 11-month-old mouse. Note also an isolated gold particle in the ER. **B:** Perikaryon in CA1 region of hippocampus of a normal young rat; note Aβ42 immunogold labeling of two MVBs adjacent to a larger unlabelled endosome. Occasional gold particles also stained small clear vesicles and at times, gold particles are found in poorly identifiable locations. **Inset** demonstrates an example of MVB Aβ42 staining in another hippocampal perikaryon of rat brain; this MVB contains several intravesicular vesicles (arrow) that are characteristic of MVBs. **C:** Aβ42 immunoreactivity in a MVB of a normal human perikaryon derived from a rapidly fixed specimen of cerebral cortex from a 44-year-old with surgical resection in the setting of an acute aneurysm. **D:** Monoclonal anti-Aβ42 antibody MBC42 does not cross-react with APP CTFs in APP Swedish-transfected N2a cells (top) or Tg2576 mouse brain (bottom). For N2a cells, total Aβ (left) or Aβ42 (right) are shown in lysate (L) and conditioned media (M). Only the antibody directed at the mid-portion of total Aβ (4G8) but not anti-Aβ42 (MBC42) reacts against APP CTFs. The lack of MBC42 staining of CTFs is also demonstrated by this immunoprecipitation/Western blot of 12-month-old Tg2576 mouse brain, where Aβ42 is evident but not CTFs or full-length APP (flAPP). IgG bands represent nonspecific immunoglobulin bands observed as a results of the IP/Western blot. **E:** Dual-labeling immuno-EM showing full-length APP and APP CTFs (using C-terminal APP antibody 369) with dark immunoperoxidase reaction product primarily in Golgi, and immunogold labeling of Aβ42 (using monoclonal antibody MBC42) demonstrating labeling of an MVB (arrow) close to Golgi in this neuron from mouse cerebral cortex. Abbreviations: MVB, multivesicular body; Golgi, Golgi apparatus; ER, endoplasmic reticulum; mit, mitochondrion; Endo, endosome; N, nucleus. Scale bars: 300 nm (A–C); 500 nm (E).

analogous increases of brain Aβ (Figure 3A) as reported by ELISA, including preplaque between 3 and 9 months of age. To determine the location of Aβ increases, mice were perfused at different ages ranging from 2 months to

20 months ($n = 16$) and processed for immunogold EM localization of Aβ42. Relatively less Aβ42 was found in 2- to 4-month-old mice. In contrast, significantly greater amounts of intraneuronal Aβ42 were found in 10-month-

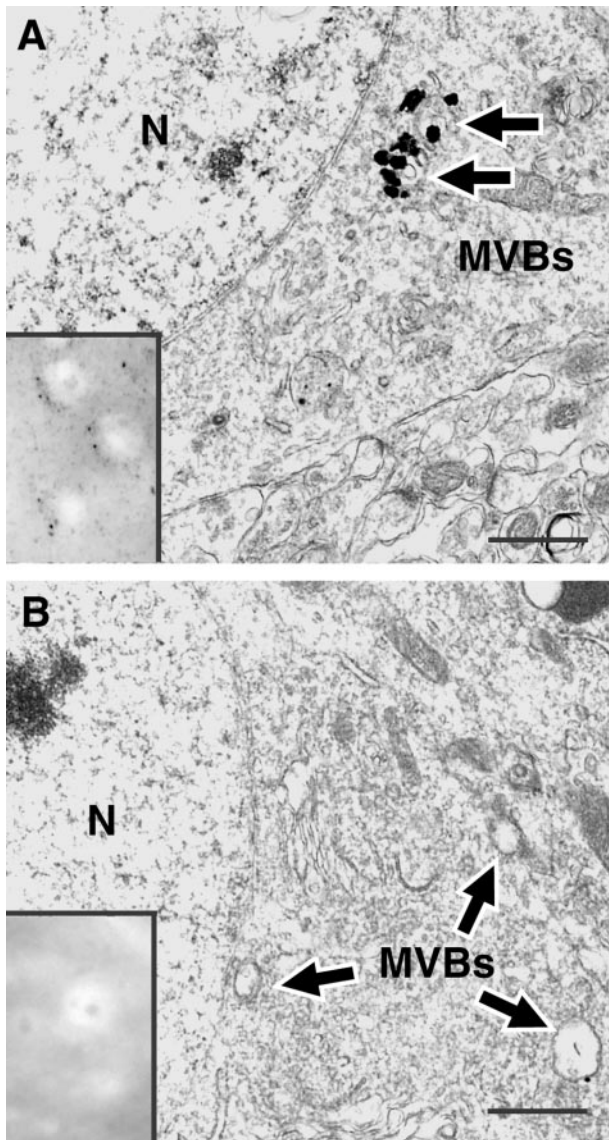


Figure 2. Absence of intraneuronal A β 42 in APP knockout mice. **A:** Immunogold labeling of several MVBs (arrows) in a neuronal perikaryon from a wild-type mouse brain. **B:** Absence of immunogold labeling of MVBs in a neuron from an APP knockout mouse. **A** and **B** were CA1 hippocampal sections from 12-month-old littermates processed in parallel. **Insets** in **A** and **B** represent lower power views to emphasize the lack of A β 42 immunoreactivity in several neurons of APP knockout mouse brain; A β 42 gold particles within several neuronal perikarya from wild-type mouse are evident (**inset** in **A**) compared with lack of gold particles in several neurons of a knockout mouse (**inset** in **B**) processed in parallel. **Arrows** indicate MVBs. Abbreviations: MVBs, multivesicular bodies; N, nucleus. Scale bars, 1 μ m.

old mice before the onset of plaque pathology (Figure 3, B and C). The A β 42 increases were most prominent on MVBs, particularly at nerve terminals (presynaptic and especially postsynaptic compartments). The immunogold labeling of A β 42 with aging in brain tissue was quantified from representative young (2-month-old) and older (10-month-old) Tg2576 mice. The MVB-associated gold particles in neuronal cell bodies ($n = 20$ each, from young and old) or processes of cerebral cortex were counted. Neuronal soma from 2-month-old mice had 1.48 ± 0.3 gold particles per MVB whereas neurons from older mice had 2.02 ± 0.4 gold particles per MVB (Figure

3D). This increase of MVB-associated gold particles in neuronal soma with aging did not reach statistical significance. A β 42 increases were most pronounced within neuronal processes (profiles), especially small dendrites ($<1.5 \mu$ m in diameter) and axon terminals. Thirty images of dendritic profiles were taken each from 2-month-old and from 10-month-old mice at random at the same magnification. The number of gold particles per MVB in processes was 1.50 ± 0.29 and 3.42 ± 0.45 for 2- and 10-month-old mice, respectively (Figure 3D; $P < 0.033$). MVB-associated gold particles were only rarely found in dendrites of 2-month-old mice. In contrast, numerous MVBs labeled with several gold particles were found in 10-month-old mouse brain. No MVBs with three or more gold particles were observed in 2-month-old dendritic profiles, whereas nine MVBs with three or more gold particles were observed in 10-month-old processes (Figure 3E). Lack of a specific marker for MVBs precludes biochemical quantification for potential changes in number of MVBs with aging in Tg2576 mice. Immuno-EM is not an optimal method for quantification and counting of MVBs by EM is further confounded by our observation that MVBs in distal processes of 10-month-old Tg2576 mice tended to be larger and contained a greater number of intravesicular vesicles than in processes of 2-month-old mice (compare Figure 3, B and C). To reduce potential bias from gold particles, we quantified total MVBs in dendrites based on morphology by EM without immunogold labeling. The number of MVBs almost doubled in dendrites of young 2-month-old compared to older 10-month-old preplaque Tg2576 mice (1.96-fold increase; $P < 0.03$).

A β 42 Accumulation within Nerve Terminals Is Associated with Neuropathology

Before the development of AD plaque pathology, we observed accumulation of A β 42 presynaptically within axon terminals and especially postsynaptically within distal dendrites. At 10 months of age and before A β plaques, dendrites were found that displayed multiple gold particles and morphological alterations, such as atypically dark A β 42 immunogold-associated MVBs, unusual clusters of ER in the vicinity of the A β 42 immunogold-associated MVBs, and abnormally collapsed, dark membranes consistent with degenerating processes in the vicinity of especially numerous gold particles (Figure 4A). At later ages, when plaques were forming, A β 42-accumulating processes were more numerous and showed strikingly abnormal morphological changes in the absence of an associated A β plaque (Figure 4; B, C, and D). Increased intraneuronal A β 42 immunogold labeling often was affiliated with swollen processes, sometimes within areas lacking normal appearing organelles or including dark structures resembling degeneration (Figure 4; B, C, and D). A β 42 accumulation could also be seen associated with disrupted appearing MVBs (Figure 4B). The presence of abnormal appearing APP-containing neurites within plaques has been known³⁷ and isolated A β fibrils have been noted in such dystrophic neu-

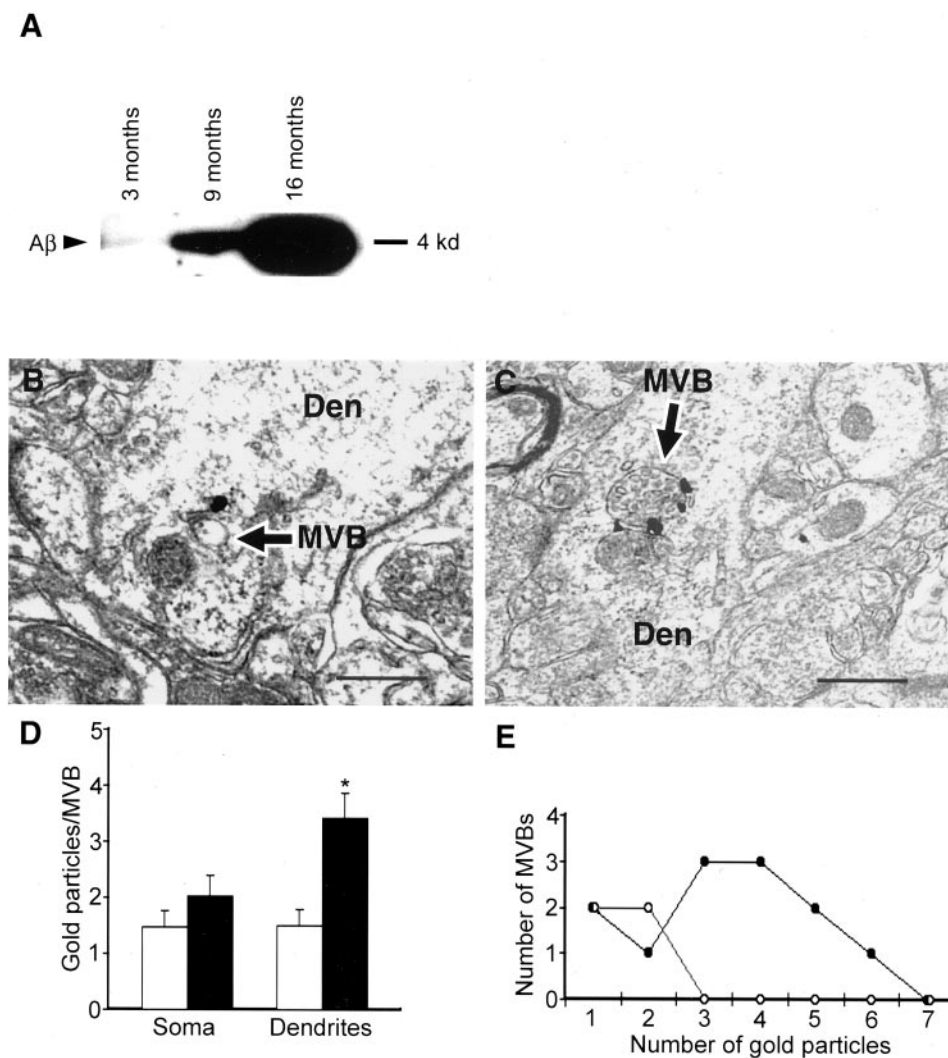


Figure 3. Neuronal A β 42 immunoreactivity increases with aging in the brains of Tg2576 mice containing the APP Swedish 670/671 FAD mutation. **A:** Immunoprecipitation and Western blot of 4-kd human A β in equivalent brain tissue (parietal cortex) of Tg2576 mice at 3, 9, and 16 months. Note the total A β increases in preplaque mouse brains between 3 and 9 months. **B** and **C:** A β 42 immunogold MVB labeling (arrows) of representative dendrites from young (2 months; **B**) and older preplaque (10 months; **C**) Tg2576 mice. Abbreviations: Den, dendrite; MVB, multivesicular body. **D:** Bar graph indicating the number of gold particles per MVB in neuronal soma (left) or dendrites (right) in 2-month-old (open bars) and 10-month-old (filled bars) Tg2576 mice (asterisk denotes statistical significance). **E:** Frequency distribution of total number of MVBs containing various numbers of gold particles in distal dendritic processes of young (2 to 3 months, open circles) and old (10 months, filled circles) Tg2576 mice. Scale bars, 500 nm (**A–C**).

rites,³⁸ but this is the first study showing that A β 42 accumulates in synaptic compartments and is associated with morphological changes. Interestingly, in a recent study isolated neurons in Down's syndrome brain with marked intraneuronal A β 42 accumulation were reported to display concomitant terminal dUTP nick-end labeling staining indicative of apoptosis.²⁶ In contrast to A β 42, A β 40 increases were less evident in A β 42-accumulating neurites, but A β 40 was seen to accumulate in A β extracellular plaques and vessel walls (data not shown).

In human AD cortical brain tissue ($n = 2$, ages 54 and 62), as in the case of the transgenic mice, we observed A β 42 accumulation especially within neuronal processes and associated abnormal morphological changes (Figure 5). Compared with non-AD cortical biopsy tissue, AD cortex had marked increases of A β 42 immunogold particles both in soma and processes, even including areas

not directly associated with A β plaques. MVBs with 5 to 20 gold particles, not seen in normal human brain, could be seen in human AD brain (Figure 5B). In AD brain, A β 42 immunogold accumulation was seen in disrupted swollen dendritic profiles displaying degenerative changes (Figure 5C). In AD and older plaque bearing Tg2576 mouse brain, astrocytes and microglia in the vicinity of A β plaques contained A β 42; this A β 42 was found especially within bundles of intermediate filaments in fibrous astrocytes (data not shown).

Discussion

This study demonstrates that MVBs are the major sub-cellular site of accumulation of A β 42 within neurons. We provide data supporting a pathogenic role for intraneuronally accumulating A β 42 because in Tg2576 mice and

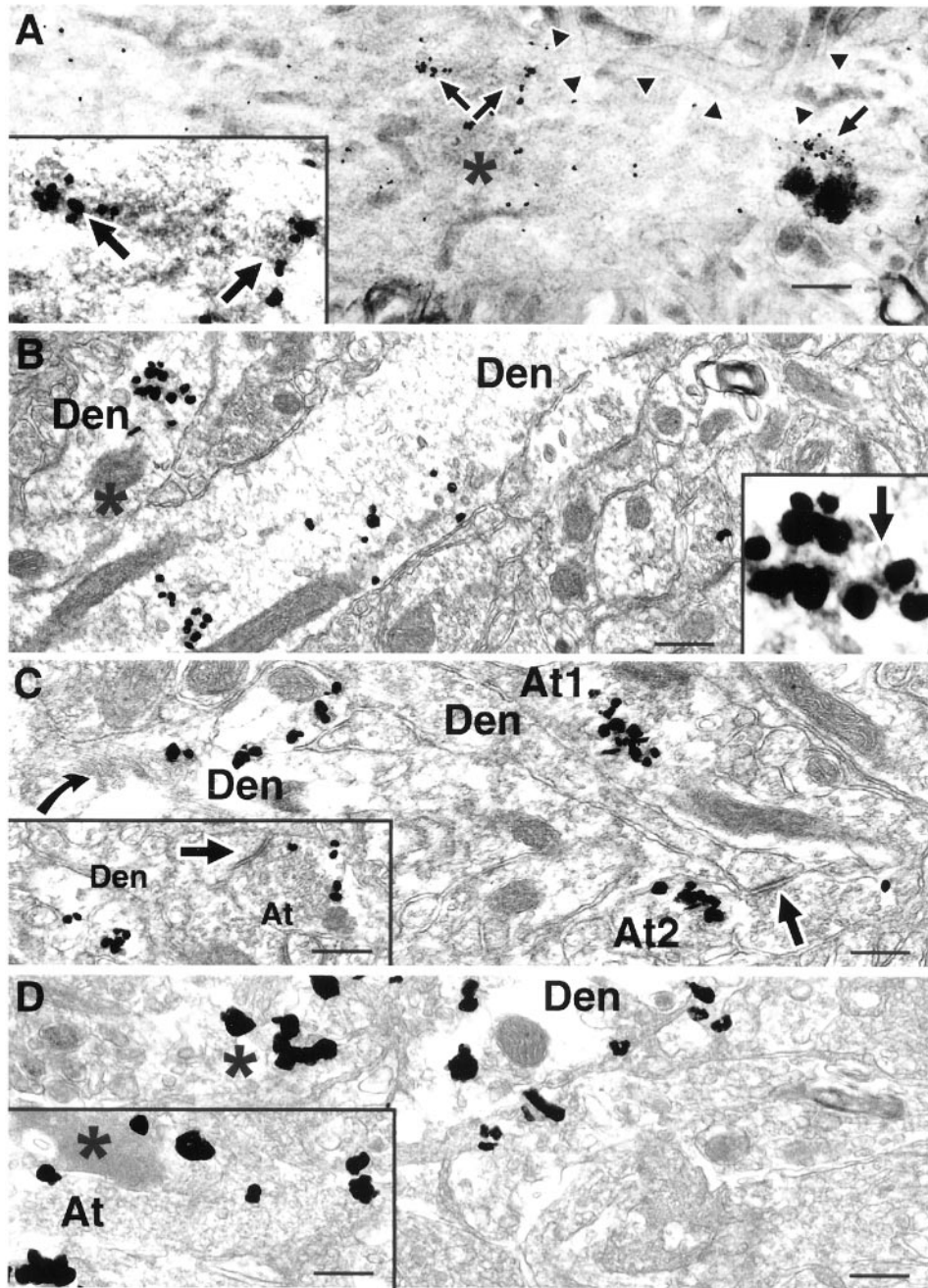


Figure 4. A β 42 accumulates within neuronal processes in Tg2576 mice before plaque formation. **A:** A dendritic process with significant MVB-associated A β 42 shows several apparent morphological abnormalities in the cortex of a preplaque 10-month-old mouse (arrowheads indicate outer membrane of this dendrite). Increased A β 42 immunogold labeling is seen within this dendrite, which, based on its relative darkness (asterisk), appears to be degenerating. Unusual images of A β 42 MVBs associated with lysosomes (such as the MVB under the single arrow at the far right) and abnormally dark A β 42 immunogold MVBs (see two MVBs in inset and MVB under the single arrow) are observed. Inset is higher magnification of two darker appearing A β 42 immunogold-associated MVBs above the paired arrows. **B:** In a 17-month-old mouse (B to D), a distended dendritic profile traversing the image with A β 42 immunogold, and absence of normal cytoskeletal organelles. A second dendrite with A β 42 is seen at the left with an abnormal dense body (asterisk) close to multiple MVB-associated gold particles; inset is a higher magnification of this MVB, in which characteristic small intravesicular vesicles can be seen (arrow). **C:** Axon terminal (At1) with substantial A β 42 immunogold and some electron-dense components suggestive of degeneration. Another axon terminal (At2) with A β 42 accumulation close to a synapse (arrow) with a dendritic spine. At left is a dendrite with abnormally stacked membranes (curved arrow) and A β 42 accumulation. Inset shows increase in A β 42 immunogold within dendritic and axonal compartments close to another synaptic density (arrow). **D:** At left, is a poorly defined process (asterisk) with A β 42 accumulation also containing clusters of abnormal, darker membranes. Inset shows degenerating dark profiles (asterisk) seemingly within an axon terminal that contains clusters of A β 42 gold labeling. Abbreviations: Den, dendrite; At, axon terminal. Scale bars, 300 nm.

in AD brain it is directly associated with abnormal cellular morphology. The finding that A β 42 accumulation and early cellular pathology occurs specifically in distal processes and at synapses could explain observations of

early decreases in synaptic markers in preplaque AD transgenic mice.¹² The observations that substantial A β accumulates within synaptic compartments, and is associated with cellular pathology, provides a molecular basis

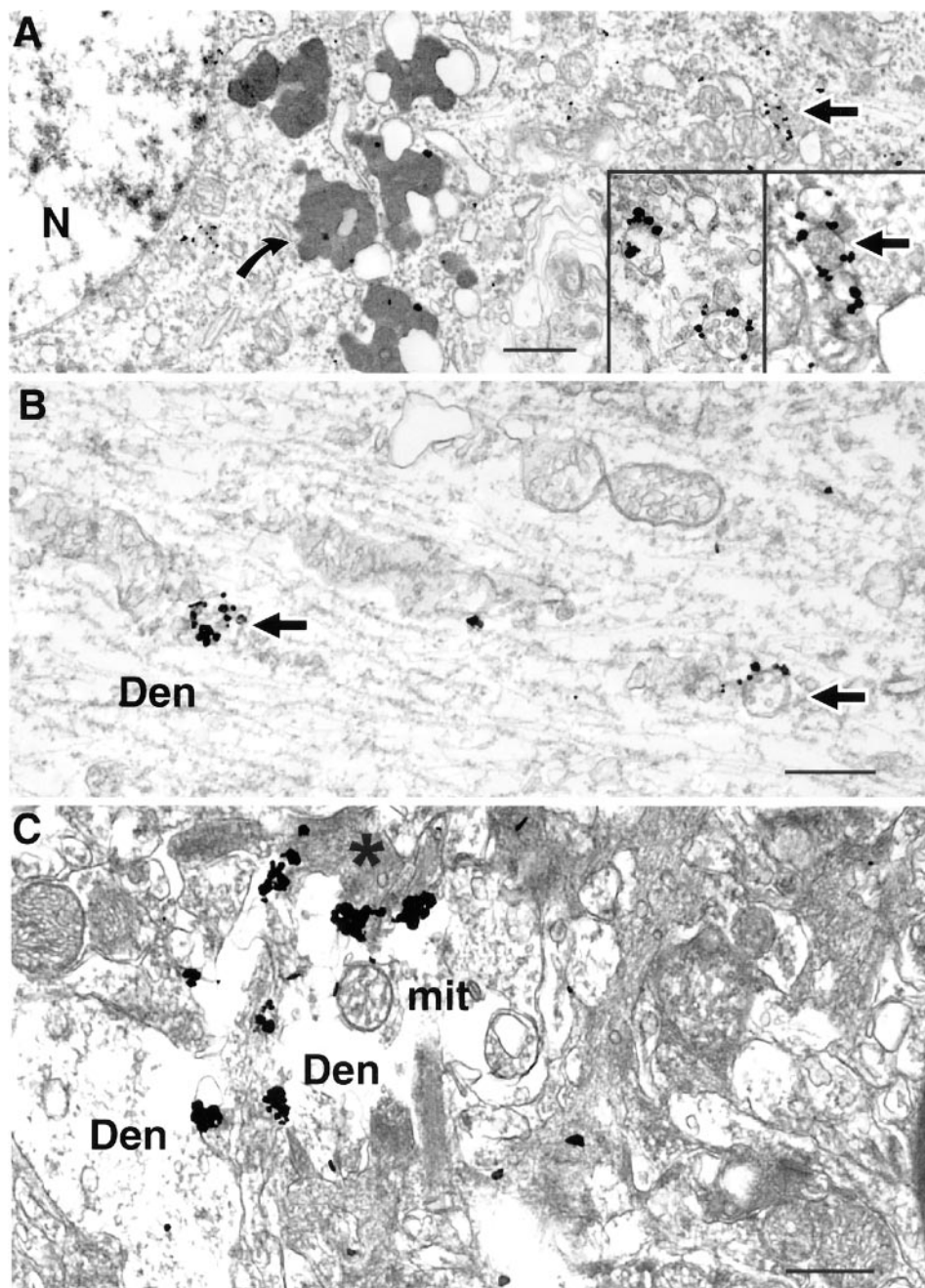


Figure 5. A β 42 accumulates within neuronal soma and processes in human AD brain. **A:** Human AD cortical brain specimen showing substantial amounts of A β 42 labeling within MVBs of an abnormal appearing neuronal soma (**arrow**); **curved arrow** indicates lipofuscin devoid of specific immunogold labeling. Nucleus has abnormal appearing accumulation of heterochromatin near the outer membrane. **Insets** represent higher power views of A β 42 immunogold labeling of MVBs in neuronal soma. **B:** Multiple A β 42 gold particles are associated with two MVBs in a dendrite. **C:** Multiple aggregated A β 42 immunogold particles are found within a disrupted, swollen dendrite containing electron-dense material (**asterisk**), indicative of degeneration. Abbreviations: Den, dendrite; mit, mitochondrion; N, nucleus. Scale bars: 1 μ m (**A**); 500 nm (**B** and **C**).

for the clinical observation correlating severity of dementia with markers of synaptic loss.³⁹

A β peptides were initially believed to be an abnormal and toxic by-product of APP metabolism, until it was found in the early 1990s that all cells seem to normally secrete A β .¹ A β was subsequently thought to be generated and rapidly secreted at the plasma membrane, until cell biological studies demonstrated that A β peptides can be found intracellularly³ and even increase in amount

with neuronal maturation or time in culture.^{3,18} Our observation of A β 42 associated with MVBs in normal brain raises the possibility that this small hydrophobic peptide may have a normal biological function. Even though APP has been studied intensively since its identification as the precursor to A β in the late 1980s, the normal function of APP remains unknown. APP knockout mice were not found to have an obvious abnormal phenotype,³⁶ but subsequent studies have indicated that APP knockout

neurons have decreased neurite extension in tissue culture⁴⁰ and deficits in synaptic plasticity.⁴¹ The normal presence and increases with aging of A β 42, the first A β peptide found in early diffuse plaques in Down's syndrome and AD, within neurons in Tg2576 mice and in AD brain, also in areas not adjacent to plaques, suggests that this intracellular A β may be the site of A β aggregation and neurotoxicity, subsequently leading to "extracellular" appearing A β plaques. We and others reported images of A β plaques seemingly originating from within neuronal soma in AD.^{2,22} We did not observe such images in Tg2576 mice. We hypothesize that the formation of plaques and lack of neuron cell death seen in APP FAD mutant transgenic mice may reflect A β 42 accumulation within distant neuronal processes with synaptosis and plaque formation, with lesser A β accumulation within soma, thereby avoiding cell death, possibly secondary to the absence of human τ .^{42,43}

Our study does not provide information on the NH₂-terminus of MVB-associated A β x-42, with A β Asp1 and A β Glu11 being major NH₂-terminal A β peptides generated by neurons.^{46,47} Because the earliest plaques with AD pathology have been reported to be NH₂ terminally truncated A β x-42,⁴⁶ we, and others, have hypothesized that NH₂-terminally truncated A β 42, such as A β Glu11-42, may be especially important in AD pathogenesis. This study again suggests, as did our and other previous studies,²²⁻²⁶ that A β x-42, and not A β x-40, is the major intraneuronal A β peptide in brain. Tissue culture studies and brain ELISA studies of AD and Tg2576 mouse brain demonstrate considerable levels of both A β 40 and A β 42 peptides. Brain A β 40 may either not be visualized as well by immunohistochemical methods or may mainly reflect that associated with blood vessels and A β plaques. In the present study, some A β 40 immunogold labeling, although less apparent than A β 42, was observed on neuronal MVBs in brain with aging.

Relatively little is known about the biological function of MVBs, which are defined by their ultrastructural appearance on EM. No clear distinction exists between MVBs and endosomes, except that MVBs tend to be regarded as smaller than, and potentially even be derived from, endosomes. In normal brain, A β 42 appeared to localize especially to small MVBs, but with aging A β 42 could also be observed on larger MVB/endosomes. MVBs are generally viewed as part of the endosomal/lysosomal system and both early and, more typically, late endosomes have been described as MVBs.^{47,48} A β is known to be generated in the endosomal-lysosomal system^{10,11} and early lysosomal abnormalities in AD have been described.^{49,50} APP metabolism within Niemann-Pick cells demonstrated a pool of accumulating intracellular A β 42 in endosomes that is differentially regulated from the constitutive A β secretory pathway.^{51,52} Possibly this pool of A β 42 represents the MVB-associated pool of A β 42. The ER has been described as an important site of A β 42 formation and we observed some A β 42 in ER of neurons in brain. Indeed, MVBs are often seen in the vicinity of the ER and may represent extensions of smooth ER.³² Similarly, the Golgi is a major site of A β generation in the secretory pathway and MVBs also are Golgi associated (Figure

1E).³² We also observed A β 42 in very small vesicles that may represent recycling vesicles between MVBs and major intracellular organelles and/or the plasma membrane. With A β 42 accumulation on MVBs with aging and AD pathology, structures representing disrupted MVBs were observed (Figure 4B). Because pre-embedding immuno-EM generally underestimates actual quantity of antigen, such MVB disruption could lead to release of enough A β 42 to induce neurotoxicity. Specifically A β 42 treatment of cells was reported to induce leakage of lysosomal contents into the cytoplasm.⁵³ Recently, it was demonstrated that A β 1-42, but not A β 1-40 or A β 42-1, is exquisitely neurotoxic when introduced into the cytosol of cultured primary neurons.⁵⁴ Thus, leakage of A β 42 from MVBs into the cytosol may be a critical neurotoxic event in AD.

The mechanism whereby intracellular A β accumulates, aggregates, and is neurotoxic to neurons remains to be elucidated. Our studies suggest but do not prove that A β 42 is the toxic entity. It was recently reported that BACE, presenilin 1, and APP are all transported along axons in membrane vesicles that require APP/kinesin-1 interactions and that vesicle preparations from peripheral nerves can generate A β .⁵⁵ Based on these findings and the fact that these vesicles also carry additional vital cargo proteins, such as the neurotrophin receptor, TrkA, Kamal and colleagues⁵⁵ hypothesized that loss of trophic support to distal synapses from aberrant A β accumulation or altered APP function could hold up this cargo and lead to neurodegeneration in AD.^{55,56} A neuron-specific C-terminal type kinesin superfamily protein (KIFC2) was found to be associated with MVBs by immuno-EM and to be important for dendritic transport of MVBs.⁵⁷ Our study supports the scenario that accumulating A β 42 located on the outer membrane of MVBs could directly associate and disrupt kinesin (and possibly even τ)-mediated transport of MVBs.

Intracellular aggregation of proteins is increasingly being linked to neurodegenerative diseases,⁵⁸ including AD. FAD mutations increase not only secreted, but also intracellular A β 42.^{59,60} Recently, a FAD mutation was discovered that leads not to increased but decreased levels of A β secretion, and to increased generation of protofibrils, leading the authors to suggest that intracellular A β increases may be especially important for AD.⁶¹ The present study supports the concept that intracellular A β is important in the genesis of AD. Previous experiments indicated that radiolabeled A β 42 can be internalized by neurons,⁶² suggesting that one route of intraneuronal A β may be via neuronal uptake and subsequent intracellular accumulation. A recent study demonstrated that epidermal growth factor and transforming growth factor- α internalize after binding to the epidermal growth factor receptor initially via clathrin-coated vesicles, but at 30 minutes of chase are found on the outer membrane of MVBs,⁶³ analogous to the location of A β 42 on the outside membrane of MVBs. Thus, MVB-associated A β 42 may be derived from endocytosis at the plasma membrane. Internally generated A β might be expected to be prominent at synapses, because APP, and therefore A β , is transported down axons⁶⁴ and dendrites.⁶⁵ Indeed, the A β domain of

APP appears critical for this sorting of APP for transport.⁶⁵ Progressive accumulation and aggregation of intracellular A β 42 in dendrites/distal processes and presynaptic and postsynaptic compartments could lead to dystrophic neurite swellings, further APP conversion to A β 42 within these processes, release of this A β 42 from normal compartments, disruption of distal processes and synapses, and then eventually to dissolution of processes/synapses with the emergence of "extracellular" A β plaques. The mechanism(s) by which A β 42 increases on MVBs, aggregates, and becomes neurotoxic within neurons and their processes, remain(s) to be determined. A better understanding of the biogenesis and biological role(s) of MVBs and a more detailed understanding of the subcellular events involved in A β -induced AD neuropathology may be of value in the development of more effective therapies for AD.

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