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Subcellular Topography of Neuronal Aβ Peptide in APPxPS1 Transgenic Mice

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In transgenic mice expressing human mutant β-amyloid precursor protein (APP) and mutant presenilin-1 (PS1), Aβ antibodies labeled granules, about 1 μm in diameter, in the perikaryon of neurons clustered in the isocortex, hippocampus, amygdala, thalamus, and brainstem. The granules were present before the onset of Aβ deposits; their number increased up to 9 months and decreased in 15-month-old animals. They were immunostained by antibodies against Aβ 40, Aβ 42, and APP C-terminal region. In double immunofluorescence experiments, the intracellular Aβ co-localized with lysosome markers and less frequently with MG160, a Golgi marker. Aβ accumulation correlated with an increased volume of lysosomes and Golgi apparatus, while the volume of endoplasmic reticulum and early endosomes did not change. Some granules were immunolabeled with an antibody against flotillin-1, a raft marker. At electron microscopy, Aβ, APP-C terminal, cathepsin D, and flotillin-1 epitopes were found in the lumen of multivesicular bodies. This study shows that Aβ peptide and APP C-terminal region accumulate in multivesicular bodies containing lysosomal enzymes, while APP N-terminus is excluded from them. Multivesicular bodies could secondarily liberate their content in the extracellular space as suggested by the association of cathepsin D with Aβ peptide in the extracellular space. *(Am J Pathol 2004, 165:1465–1477)*

One of the pathological hallmarks of Alzheimer's disease (AD) is the extracellular deposition of β-amyloid (Aβ) peptides.[1,2] The Aβ peptide originates from the proteolytic processing of single-pass transmembrane proteins, the β-amyloid precursor proteins (APP).[3,4] The Aβ peptide ends at amino acid 40 or 42 and may be N-truncated.[5,6] Aβ peptide may be produced from APP in the endoplasmic reticulum (ER),[7,8] in post-ER compartments[9,10] or in the *trans*-Golgi network[11,12] and targeted to secretion vesicles, the so-called "secretory pathway."

Alternatively, APP can be internalized from the cell surface through the endocytic pathway and directed toward the endosomal-lysosomal system.[13–15] Impeding endocytosis decreases Aβ production.[16] Abnormal early endosomes are detected in sporadic Alzheimer disease, Down syndrome, and in cellular models of Niemann-Pick type C in which Aβ peptide accumulates intracellularly.[17–20] Overexpression of cation-dependent mannose 6-phosphate receptor, a molecule involved in the transport of hydrolases toward the endosomal-lysosomal system, dramatically increases the secretion of Aβ 40 and Aβ 42 in the culture medium.[21] In the same way, Rab5-stimulated up-regulation of the endocytic pathway increases intracellular APP β-cleavage and Aβ peptide production.[22] Where the γ-cleavage occurs in the endosomal-lysosomal pathway, remains controversial: it could take place in lipid rafts,[23] located at the cellular membrane,[24] where Aβ starts to accumulate[25] or in the lysosome itself, which contains the necessary enzymatic machinery.[26]

Accumulation of Aβ peptide within neurons has been documented in AD,[27,28] particularly in trisomy-21.[29,30] The intracellular accumulation was maximal at early stages of the disease and decreased thereafter. It occurs in multivesicular bodies, within presynaptic and especially postsynaptic compartments, in APP transgenic mice and in AD.[31] The multivesicular body (MVB) belongs to the late endosome compartment and fuses with lysosomes. The lumen of this ovoid or spherical organelle contains membrane-bound vesicles. MVB is a highly con-

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Table 1. Genotype, Age, and Number of Animals

Genotype Age (months)	WT	PS1	APP	APPxPS1
2				1
3		2		4
5	2	2	2	2
6		2		4
7		2		2
9	2	3	3	5
11				1
12	2	2		4
15				1
Total	6	13	5	24

served compartment that helps regulating and degrading transmembrane proteins. These proteins initially located in the membrane of the endosome are secondarily targeted to the endoluminal vesicles (for review, reference 32). Accumulation of APP has been shown in MVB–like organelles in cultured leptomeningeal smooth muscle cells and brain pericytes.[33]

Aβ peptide also accumulates in the neuronal cell body of APPxPS1 transgenic mice, when they are young, and disappears in aged animals.[34–36] We studied the time course of Aβ intracellular accumulation and characterized the intracellular compartments containing Aβ-immunoreactive material using double immunofluorescence and electron microscopy. We investigated brain samples from transgenic mice expressing human-mutated APP (APP_{751} with both the Swedish and the London mutation) and human PS1 bearing the M146L mutation.[36] Double immunofluorescent labeling with a combination of antibodies against Aβ, APP, and different organelle markers were examined with confocal microscopy and the volume occupied by the various organelles was evaluated. The intracellular localization of the Aβ peptide was determined at the ultrastructural level by immunogold electron microscopy.

Materials and Methods

Transgenic Mice and Tissue Preparation

Generation and characterization of single APP_{751} with the Swedish and London mutation (APP_{751}SL) and double APP_{751}SLxPS1M146L transgenic mice were described previously.[34–37] In these animals, APP is expressed at a high level in all cortical neurons under the control of the Thy-1 promoter. Human PS1 with the M146L mutation is expressed under the control of the HMG-CoA reductase promoter. The level of amyloid load was found to be quite reproducible at a given age; the coefficient of error between animals is below 10%.[36] PS1 single transgenic mice, which do not develop amyloid plaques, and wild-type animals were also examined.

Intracytoplasmic Aβ accumulation was searched for in 24 APPxPS1 mice, five APP single transgenic, 13 PS1 single transgenic mice, and six wild-type animals of the same genetic background (C57Bl6) (Table 1). Animals were handled by Transgenic Services Department Charles River Laboratories France, and handled according to the French guidelines for animal care.

Brain Tissue Preparation for Morphological Analysis

Animals (4 wild-type mice, 13 PS1, 4 APP, and 22 APPxPS1 transgenic mice from 2 to 15 months of age, Table 1) were sacrificed by intracardial perfusion with 4% paraformaldehyde (PFA) in phosphate- buffered saline (PBS), pH 7.4. Brains were removed and post-fixed for 1 hour in PFA at 4°C. Coronal sections (2-mm thick) were made, sectioned with a freezing microtome or embedded in paraffin. For electron microscopy, three additional 9-month-old animals (one APP, one APPxPS1, and one wild-type) were used. Post-fixation in 2.5% glutaraldehyde for 24 hours was added to the previous protocol.

Brain Tissue Preparation for Western Blot Analysis

For Western blot analysis, crude brain homogenates were used. Snap-frozen cerebral hemispheres from one wild-type and one APPxPS1 (9- month-old animals) were weighed and homogenized on ice in 10 volumes (w/v) of buffer containing 0.32 mol/L sucrose, 4 mmol/L Tris-HCl, pH 7.4 and a protein inhibitor cocktail (Complete, Roche Diagnostics GmbH, Mannheim, Germany). Protein concentration was determined by BCA protein assay (Pierce, Rockford, IL).

Western Blot Analysis

Proteins from crude brain homogenates (10 μg) were mixed with Laemmli sample buffer, then separated by electrophoresis on 12% Tris-HCl polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane (0.45 μm, Amersham, France), blocked with 5% (w/v) nonfat dry milk in TBST (50 mmol/L Tris-HCl (pH 8.1), 150 mmol/L NaCl, 0.05% (v/v) Tween 20), and incubated overnight at 4°C with the primary antibody. The following primary antibodies were applied to the membrane (Table 2): anti-APP Nter 1:2000, anti-APP Cter (737–751) 1:1000, anti-APP Cter (705–751) 1:3000, and anti-$A\beta_{8-17}$ 1:500 in 5% milk TBST. Binding of the primary antibody was detected with a horseradish peroxidase-conjugated secondary antibody at a 1:5000 dilution followed by the enhanced chemiluminescence detection system (ECL, Amersham, France) according to the manufacturer's instructions.

Histology

Hematoxylin-eosin (H&E) and Bodian silver stains were routinely performed. To demonstrate the amyloid nature

Table 2. Antibodies

Antibody	Dilution	Immunogen	Source	Labeling
APP-Cter rabbit polyclonal	1/2000	Synthetic peptide conjugated to KLH corresponding to aa 705–751 of the huAPP751	B. Allinquant, INSERM U573, Paris, France	C-terminal region of APP
APP-Cter goat polyclonal	1/100	Synthetic peptide conjugated to BSA, corresponding to aa 737–751 of the huAPP751	Abcam, Cambridge, UK #2083	C-terminal region of APP
APP Nter mouse monoclonal (clone 22C11)	1/200	purified recombinant Alzheimer precursor APP695 fusion protein	Chemicon International, distributed by Euromedex, Souffelweyersheim, France	N-terminal region of APP (aa 66–81)
β-amyloid (Aβ) mouse monoclonal (clone 6F/D3)	1/200	KLH-aa 8–17 from human Aβ peptide	Dako Corporation, Glostrup, Denmark	Aβ peptide 40 & 42
β-amyloid (Aβ) rabbit polyclonal	1/100	Synthetic β-amyloid peptide 1–40 conjugated to BSA	Chemicon International, Temecula, CA, USA	Aβ peptide 40 & 42
β-amyloid (Aβ) E50 Rabbit polyclonal	1/100	Aa 17–31 human Aβ peptide	H. Akiyama, Institute of Psychiatry, Tokyo, Japan	Aβ peptide 40 & 42
β-amyloid (Aβ) FCA 18 rabbit polyclonal	1/500	KLH-aa 1–8 from human Aβ peptide KLH-DAEFRHDS-Cys	F. Checler Sofia Antipolis, Nice, France	N terminal part of Aβ
β-amyloid (Aβ) FCA 3340 rabbit polyclonal	1/50	Aa 33–40 human Aβ peptide KLH-Cys-GLMVGGVV	F. Checler Sofia Antipolis, Nice, France	Aβ 40
β-amyloid (Aβ) FCA 3542 rabbit polyclonal	1/50	Aa 35–42 human Aβ peptide KLH-Cys-MVGGVVIA	F. Checler Sofia Antipolis, Nice, France	Aβ 42
Bip (Grp78) rabbit polyclonal	1/200	KLH-aa 645–654 rat Grp78	StressGen Biotechnologies Corporation, Victoria	Endoplasmic reticulum
Golgi rabbit polyclonal	1/1000	MG-160 sialoglycoprotein	N. Gonatas, University of Pennsylvania, Philadelphia, USA	Golgi apparatus
Cathepsin D rabbit polyclonal	1/1000	Human liver active cathepsin D	Dako Corporation, Glostrup, Denmark	Enriched in lysosome lumen
LAMP2 rabbit polyclonal	1/200	aa 1–207 of human Lamp2	Santa Cruz, Santa Cruz, USA	Lysosome membrane
Early endosomal antigen 1 rabbit polyclonal	1/250	human EEA1 aa 1391–1410	Affinity Bioreagents, Golden, USA	Early endosomes
Cox 2 rabbit polyclonal	1/500	subunit 2 of human mitochondrial complex IV	A. Lombès, Inserm U153, Paris	Mitochondria
Flotillin-1 mouse monoclonal	1/1000	aa 312–428 (C-terminal) of mouse flotillin-1	Translab, Erembodegem, Belgium	Protein enriched in raft
GFAP rabbit polyclonal	1/500	GFAP from cow spinal cord	Dako Corporation, Glostrup, Denmark	Astrocytes
Ubiquitin rabbit polyclonal	1/500	Ubiquitin isolated from cow erythrocytes	Dako Corporation, Glostrup, Denmark	Ubiquitinated proteins
SNAP25 mouse monoclonal, (clone SMI 81)	1/5000	synaptosome-associated protein	Sternberger, Lutherville, USA	Synapses

of the deposits, sections were stained with Congo red and thioflavin S.

Immunohistochemistry

Immunostaining was performed on 5-μm thick paraffin sections. Sections were de-paraffinized in xylene, re-hydrated through ethanol (100%, 90%, 80%, and 70%) and finally brought to water. They were microwaved twice at 400 W in citrate buffer 0.01 mol/L, pH 6.0 for 10 minutes. When immunoperoxidase was used, endogenous peroxidase activity was quenched in a TBST solution containing 0.3% H_2O_2 and 20% methanol. Non-specific binding was blocked by incubating sections for 20 minutes in 2% bovine serum albumin in TBST. Appropriate dilutions of primary antibodies (Table 2) were then applied overnight in a humidified chamber at room temperature.

Monoclonal antibody 22C11 against APP (APP_{66-81}) is commercially available. It does not cross-react with the Aβ sequence.[38,39] Rabbit polyclonal antibody against the

Table 3. Secondary Antibodies and Fluorochromes for Immunofluorescence

Fluorochrome	Specificity	Species	Absorption (nm)	Emission (nm)	Dilution	Origin
Cy3	Anti-rabbit	Goat	552	570	1/400	Jackson ImmunoResearch Laboratories
Cy2	Streptavidin conjugated (used with biotinylated anti-mouse)		492	510	Streptavidin CY2:1/400 (biotinylated anti-mouse: 1/200)	Streptavidin CY2, Jackson ImmunoResearch Laboratories; Biotinylated anti-mouse, Amersham
FITC	Anti-rabbit	Goat	492	520	1/400	Jackson ImmunoResearch Laboratories
Cy3	Anti-mouse	Goat	552	570	1/400	Jackson ImmunoResearch Laboratories

C-terminal region of APP (generous gift of B. Allinquant, Inserm U573, Centre Paul Broca, Paris, France) was generated in the rabbit using the intracellular portion corresponding to amino acids (aa) 705–751. This APP fragment does not include the Aβ peptide sequence. Another polyclonal antibody against the C-terminal APP region encompassing aa 737–751 made in the goat was purchased (Abcam). This antibody does not recognize the Aβ sequence. E50 (generous gift of H. Akiyama, Institute of Psychiatry, Tokyo, Japan) is a polyclonal antibody raised against aa 17–31 ($A\beta_{17-31}$) of human Aβ peptide. FCA 1–8 (generous gift of F. Checler, Institut de Pharmcologie Moleculaire et Cellulaire, Valbonne Sophia-Antipolis, France) recognizes the N-terminal portion of Aβ (aa 1–8), while, FCA40 and FCA42 (generous gifts of F. Checler) are specific for Aβ C terminus (including the COOH group), ending respectively at aa 40 and 42.[40] The antibodies against organelles included a rabbit antiserum against MG160 (a Golgi sialoglycoprotein), which labels Golgi *cis*- and medial *cis*-ternae[41] (generous gift of N. Gonatas, University of Pennsylvania, Philadelphia, PA, USA) and a rabbit anti-serum against Cox2, a mitochondrial enzyme (generous gift of A. Lombès, Inserm U573, Paris, France).[42] The GRP78 antibody recognizes a KDEL sequence causing retention in the ER. The antibody against cathepsin D labels the lysosomes in which it is concentrated. EEA1 (early endosome antigen 1), a 162-kd autoantigen associated with subacute cutaneous systemic lupus erythematosus, localizes to early endosomes through the zinc-binding motif FYVE and interacts with Rab-5.[43] (Table 2).

Immunoperoxidase

The appropriate biotinylated secondary antibody was applied for 30 minutes followed by 30 minutes incubation with streptavidine-peroxidase complex. The horseradish peroxidase activity was revealed with 3–3′-diaminobenzidine (DAB). The ABC-system and the peroxidase substrate were used according to the manufacturer's instructions (Chemmate K500111, Dako Corporation, Glostrup). Primary antibodies were omitted in control sections.

Immunofluorescence

Double immunofluorescence, on sections from 5- and 9-month-old animals, made use of antibodies from different species (one mouse monoclonal and one rabbit polyclonal). The secondary antibodies were directly linked with fluorescein isothiocyanate (FITC), carbocyanin 2 or 3 (Cy2 or Cy3). In some experiments, an intermediate step of streptavidin-biotin amplification was applied (Table 3). Slides were examined with a Leica confocal microscope (TCS), equipped with a krypton-argon laser (excitation wavelengths at 488, 568, and 647 nm).

Mapping of Aβ-Positive Granules and Plaques

To visualize the distribution of Aβ-positive intraneuronal granules and amyloid deposits, a series of sagittal section cut at 25-μm thickness was obtained at ages 2, 5, 9, 11, and 15 months. They were immunostained with anti-$A\beta_{8-17}$. The maps were drawn with the following apparatus, furnished by Explora Nova (La Rochelle, France) a camera, plugged in the phototube of a microscope, transmitted the microscopical image to a video screen. Linear transducers, fastened to the microscope moving stage, recorded its position and transmitted it to a PC computer. The borders of the sample and of the regions of interest were manually drawn. The profiles of the Aβ-positive deposits and of the granules containing neuronal profiles were pointed, using a mouse, on the video screen. The software (Mercator, Explora Nova, La Rochelle, France) drew the map at the appropriate scale on the video screen and kept track of the pointed profiles. A label indicated, on the video screen, the profiles, which had already been pointed to avoid counting them twice.

Quantitative Evaluation

Quantitative evaluation of Aβ co-localization with organelle markers (Grp78 for endoplasmic reticulum, MG160 for Golgi apparatus, cathepsin D for lysosomes and EEA1 for early endosomes) was performed on pictures of cortical neurons obtained with a confocal microscope using a X100 immersion oil objective. Neuronal profiles containing both Aβ-positive granules and the organelle marker were selected. For each neuronal profile, three images were analyzed. The green and red channels were acquired separately to avoid cross-talk: Aβ immunofluorescence was recorded on one channel; immunofluorescence of the organelle marker was collected on the other one. Merging these two images using a binary AND operation provided a third picture reflecting Aβ peptide co-localization with the organelle marker. The measures were performed using Image J, an image analysis program for PC, available on the Internet. (http://rsb.info.nih.gov/ij/). We assessed, after adequate thresholding, the total area of Aβ immunoreactivity within the neuron, the area occupied by the organelle and finally the area occupied by the co-localization of Aβ peptide with the organelle marker. The ratio: (area of Aβ immunoreactivity colocalized with an organelle marker)/(total area occupied by Aβ immunoreactivity) was used to estimate the volume proportion of total intracellular Aβ peptide localised in this organelle. According to Delesse principle[44], this ratio is $A_A = A_v$ (A_A = proportion of surface area; V_v = proportion of volume). For each organelle, a mean of 17 neurons could be evaluated in this way. The surface area occupied by Aβ peptide was variable from one neuron to the next. Using this variability, we tried to find out which organelle had increased its volume when the neuron had increased its content in Aβ peptide. Such a parallel increase can, indeed, be detected by a high correlation coefficient between the area of Aβ immunoreactivity and the area occupied by the organelle marker under investigation.

Immunogold Electron Microscopy

One-mm^3 blocks were excised from area corresponding to deep cortical layers and post-fixed in glutaraldehyde. Semi-thin sections, 1-μm thick, were obtained with a Reichert-Jung ultramicrotome. They were stained with toluidine blue (1%) for 2 minutes. Neurons located in plaque rich areas were selected and 90-nm thick sections were obtained. Immunogold labeling was performed according to standard post-embedding protocol.[45] Anti-$A\beta_{17-31}$, anti-C-terminal APP, anti-flotillin and anti-cathepsin D were applied in simple immunolabeling experiments. Ten-nm and 20-nm gold particles, coupled with species-specific secondary antibody, were used for double labeling experiments. The sections were incubated with the following couples of primary antibodies: APP $Cter_{705-751}$ & flotillin-1, $A\beta_{8-17}$ & cathepsin-D, $A\beta_{17-31}$ & flotillin-1. The sections were observed with a CM100 (Philips, Limeil-Brévannes, France) electron microscope.

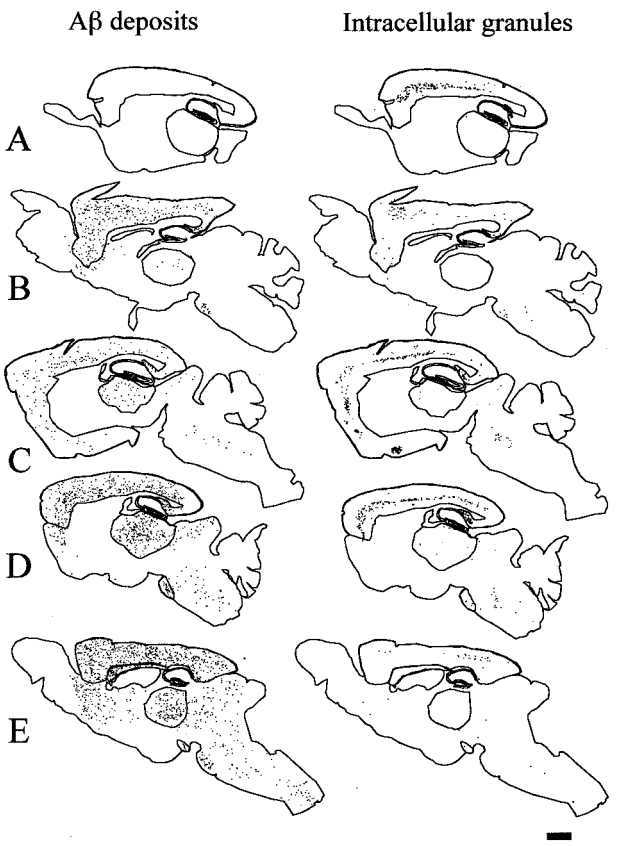

Figure 1. Maps of the extracellular Aβ deposits and Aβ-positive granules. The extracellular deposits of Aβ peptide (**left**) and the neurons containing Aβ-positive granules (**right**) were manually mapped using a dedicated software (Explora Nova, La Rochelle). Each dot is an Aβ deposit (**left**) or a granule containing neurone (**right**) **A:** 2 months; **B:** 5 months; **C:** 9 months; **D:** 11 months; **E:** 15 months. **Bar**, 1 mm.

Results

Aβ Deposits

All of the antibodies against Aβ peptide revealed extracellular deposits in APPxPS1 transgenic animals.[36] Aβ deposits were not seen at 2 months of age (Figure 1A, left). At 5 months, they were numerous in the isocortex and in the hippocampus; some were found in the thalamus; none were present in the brainstem (Figure 1B, left). At 9 months, deposits were also found in the brainstem and their density kept increasing in the isocortex, hippocampus, and thalamus (Figure 1C, left). The deposits were dense and spherical. Ill-limited, diffuse deposits, such as those observed in human aging, were never observed. The deposits affected all of the layers but were predominantly located in deep layer V and VI of the isocortex (Figure 2A). In the hippocampus, Aβ deposits mainly involved the molecular layer of the dentate gyrus, the pyramidal layer and stratum oriens of the CA1-CA3 sectors (Figure 2, A and B). The core of Aβ deposits were Congo red- and thioflavin S-positive. Occasional capillaries were labeled. Deposits in the striatum or in the cerebellum were not observed.

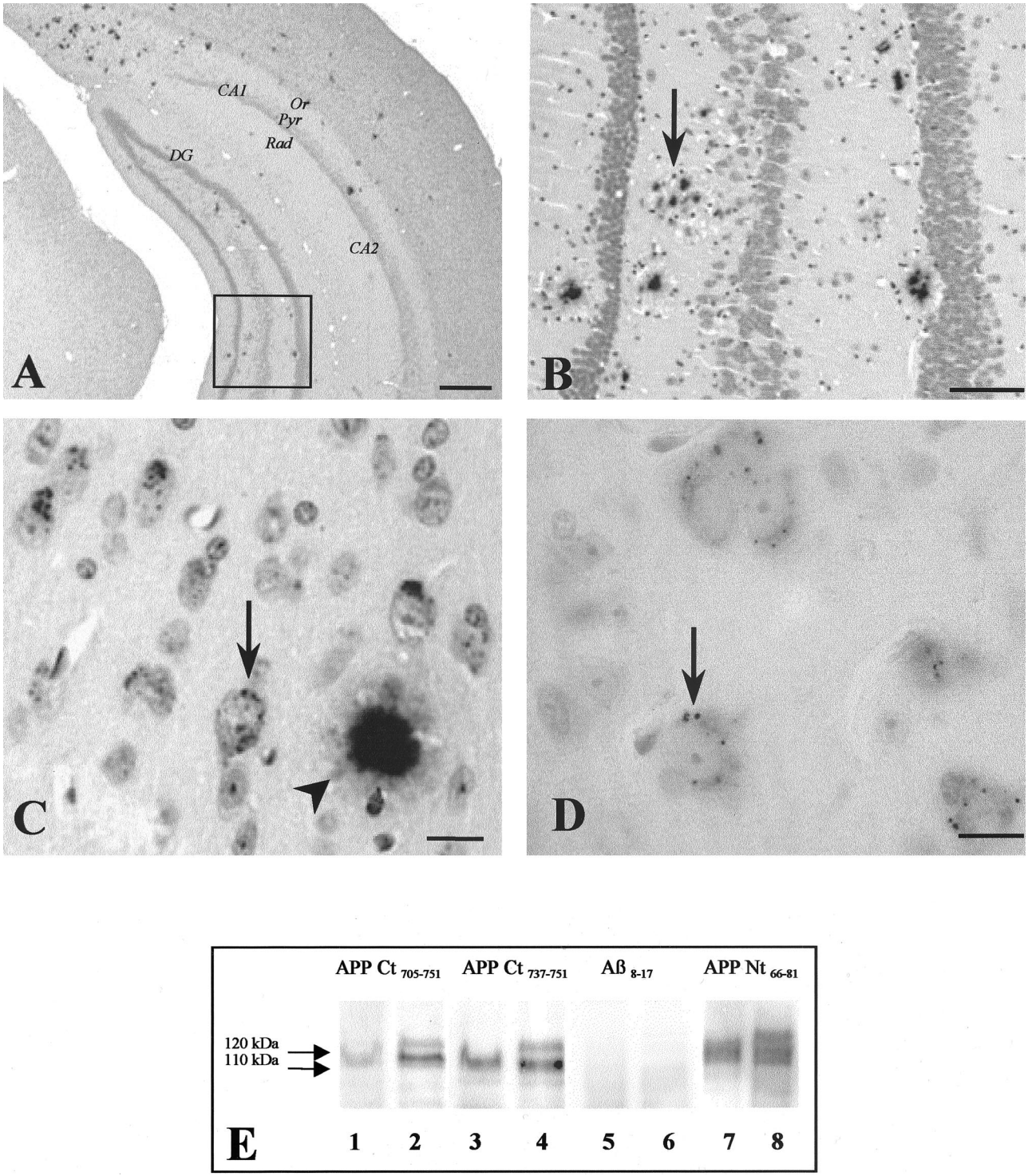

Figure 2. Immunoperoxidase $A\beta_{8-17}$ immunostaining of Thy-1 double APP_{751}SLxPS1M146L transgenic mouse (**A**, **B**, and **C**) and Thy-1 APP_{751}SL single transgenic (**D**) mouse brain sections. The mice were 9 months (**A**, **B**, and **C**) and 5 months old (D). **A:** Distribution of Aβ immunoreactive deposits. Or, stratum oriens; Pyr, stratum pyramidale; Rad, stratum radiatum. The rectangle in **A** is enlarged in **B**. **B:** Dentate gyrus corresponding to rectangle in **A**, numerous plaques are visible (the **arrow** points to one of them). **C:** Intraneuronal granules (**arrow**) close to a plaque (**arrowhead**) in APPxPS1 transgenic animal. **D:** Intraneuronal granules (**arrow**) in an APP single transgenic animal. **Bars: A**, 0,5 mm; **B**, 50 μm; **C**, 15 μm; **D**, 10 μm. **E:** Brain homogenates from one wild-type mouse (**lanes 1**, **3**, **5**, and **7**) and one Thy-1 double APP_{751}SLxPS1M146L transgenic mouse (**lanes 2**, **4**, **6**, and **8**) were analyzed by immunoblot using antibodies against APP Cter 705–751 (**lanes 1** and **2**), APP Cter 737–751 (**lanes 3** and **4**), $A\beta_{8-17}$ (**lanes 5** and **6**), and APP $Nter_{66-81}$ (**lanes 7** and **8**). Two bands (approximate molecular weight, 120 and 110 kd band, **arrows**) were detected by the antibodies to APP Cter and the antibody to APP Nter (22C11). These bands correspond to the full-length APP. The 120 kd visible in the transgenic animal corresponds to the human APP transgene. The antibody against $A\beta_{8-17}$ did not cross-react with the full-length APP, either native or transgenic (**lanes 5** and **6**).

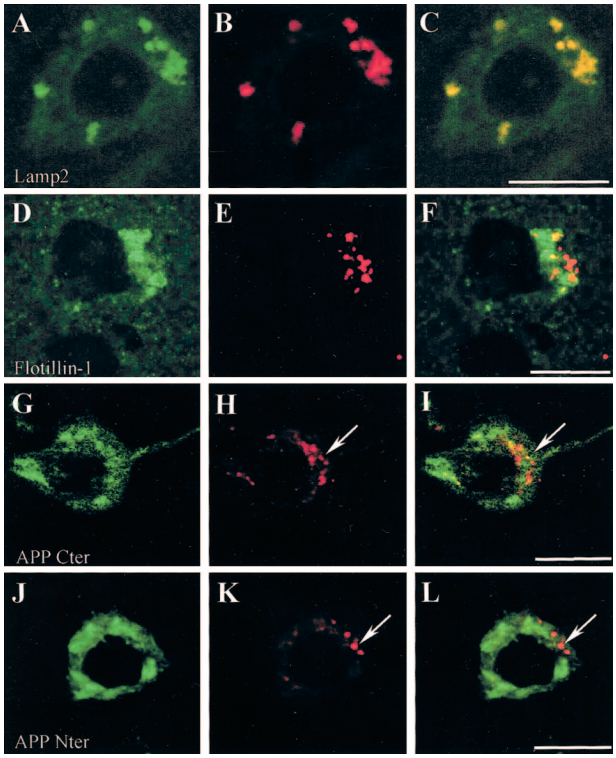

Figure 3. Double immunofluorescence examined with laser confocal microscope. Labeling with the Aβ peptide antibody is shown in red. Labeling with the other antibodies is shown in green. See Table 2 for details concerning the antibodies. Lamp2: lysosomal-associated membrane protein 2. Flotillin-1: a marker of raft and of multivesicular bodies. APP Cter, C terminus of the amyloid precursor protein (antibody raised against $APP_{705–751}$); APP Nter, N terminus of the amyloid precursor protein (antibody raised against $APP_{66–81}$). In **H** and **I**, **arrows** point to intraneuronal granules that are immunolabeled with both anti-$A\beta_{8–17}$ and APP Cter ($APP_{705–751}$) antibodies. In **K** and **L**, **arrows** point to intraneuronal granules, which are only labeled with anti-Aβ antibody ($A\beta_{1–40}$ polyclonal, Chemicon) and not with the APP Nter antibody. **Bars**: **A** to **F**, 5 μm; **G** to **L**, 10 μm.

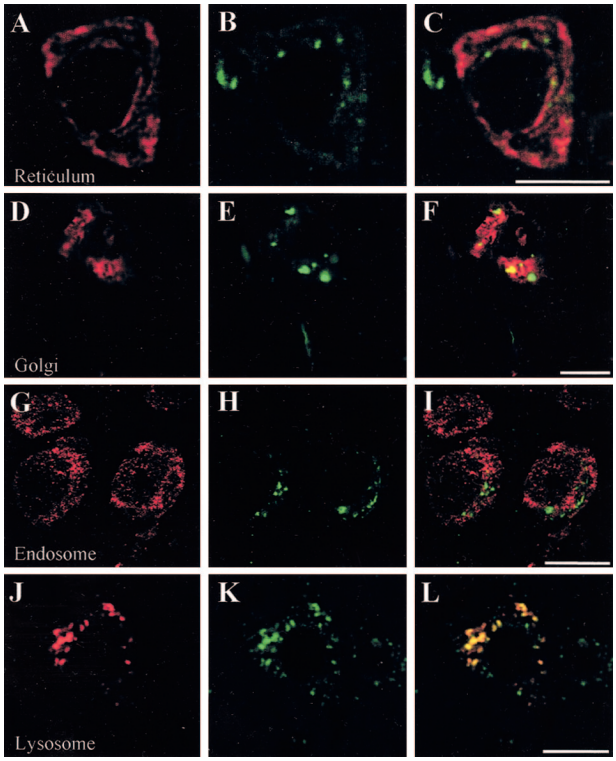

Figure 4. Double immunofluorescence examined with laser confocal microscope. Antibodies labeling organelles markers (**A**, **D**, **G**, and **J**) and $A\beta_{8–17}$ (**B**, **E**, **H**, and **K**) are visualized respectively in red and in green. See Table 2 for details concerning the antibodies. The pictures on the right (**C**, **F**, **I**, and **L**) are merged images of the green and red signals; yellow indicates colocalization. Reticulum, endoplasmic reticulum labeled by Bip/GRP78; Golgi, Golgi apparatus labeled by MG160; Endosome, labeling by the antibody directed against EEA1 (early endosome autoantigen I). Lysosomes, organelles labeled by an antibody against cathepsin D, ie, lysosomes and multivesicular bodies having merged with lysosomes. **Bars**: **A** to **C**, 5 μm; **D** to **L**, 10 μm.

Aβ-Positive Intracellular Granules

Aβ antibodies revealed spherical granules (0.5 to 1 μm in diameter), in APPxPS1 transgenic animals (Figure 2, C and D). The granules were immunolabeled by Aβ antibodies, covering the whole length of the peptide including its C- and N-termini (Table 2). They were Congo red- and thioflavin S-negative, were not autofluorescent when examined under UV light, and were not ubiquitinated.

The granules were located in the perinuclear region of the cell body and were identical in young and old animals (Figure 2, A and B). The cells containing the granules were identified as neuronal by their shape and by the presence of SNAP 25-positive synapses along their cell membrane. GFAp-positive cells, endothelial cells or pericytes never contained granules. The neurons containing Aβ-positive granules were found in isocortical layers III, V and VI, CA1-CA3 sectors of the hippocampus, subiculum, amygdaloid complex, dorsal thalamus, and brainstem. No granule containing neurons were ever observed in the striatum or in the cerebellum. Granules containing neurons were seen in the isocortex of an animal as young as 2 months of age and devoid of any extracellular deposits (Figure 1A, right). They were also seen in the hippocampus and brainstem at 5 months and, at 9 months, in the thalamus (Figure 1, B and C, right).

To determine whether the granules were related to the PS1 transgene, we also searched for granules in APP and in PS1 single transgenic mice. They were present in APP (Figure 2D), but not in PS1, single transgenic mice. They were not detected in wild-type animals.

Presence of APP C-Terminal Fragments in the Granules

The antibodies against the C-terminal region of APP (rabbit polyclonal against $APP_{705–751}$ and goat polyclonal against $APP_{737–751}$) as well as the monoclonal antibody 22C11 ($APP_{66–81}$) against the N-terminal region of APP labeled a band at 110 kd on blots of brain extracts of wild-type mice. In transgenic animals an additional band was detected at 120 kd, at the expected weight for the product of the $huAPP_{751}$ transgene (Figure 2E). The 110- and 120-kd bands were not recognized by the $A\beta_{8–17}$ antibody.

Immunohistochemistry showed that the C-terminal antibodies, anti- APP Cter 705–751 and anti-APP Cter 737–751, did not label Aβ peptide deposits but immuno-

Table 4. Co-localization of Organelle Markers and Aβ

Amyloid protein antibody	Organelle marker antibodies	Cellular compartment	Aβ co-localized/total Aβ (%)
$A\beta_{8-17}$ mAb (6FD3)	Grp78	Endoplasmic reticulum	14
	MG160	Golgi	42
	Cathepsin D	Lysosomes	57
	EEA1	Early endosomes	8
	SNAP25	Synaptic vesicles	0
	Cox2	Mitochondria	0

stained some dystrophic neurites which were not recognized by the Aβ antibodies. The antibodies against APP Cter 705–751 and APP Cter 737–751 labeled a high proportion (88 ± 7%, mean ± SEM) of Aβ-positive granules in double immunostained sections (Figure 3G). A small proportion of granules were only labeled by the APP Cter antibodies, a few were only labeled by the Aβ antibodies (Figure 3, G to I). By contrast, the monoclonal antibody 22C11 (APP_{66-81}), directed against the N-terminal part of the molecule, did not label any granule (Figure 3, J to L).

Double Labeling of the Granules with Organelle Markers

In double immunofluorescence experiments using antibodies directed against Aβ peptide and organelle markers, Aβ immunoreactive material co-localized mainly with lamp-2 (Figure 3A), a lysosomal membrane protein, cathepsin D (Figure 4J), a hydrolase usually found in lysosomes, and MG160, a marker of the Golgi apparatus (Figure 4D). There was no difference in co-localization at 5 and 9 months. Co-localization between Aβ peptide and organelle markers involved 14%, 42%, 57%, and 8% of the total intracellular Aβ, for GRP78 (endoplasmic reticulum), MG160 (Golgi apparatus), cathepsin D (lysosomes), and EEA1 (early endosomes), respectively (Table 4). The sum of the percentages of co-localization (121%) was higher than 100% because the measure could only be done on neurons where both Aβ and organelle markers immunoreactivity was present. Co-localization was, therefore, over-represented in this sample. Correlation between the area occupied by Aβ immunoreactivity in the neuron and the size of the organelles (corresponding to the area of immunoreactivity of their markers) was highly significant for lysosomes (correlation between immunoreactive areas occupied by Aβ peptide and cathepsin D: $r = 0.82$; $P < 0.0005$) and for Golgi apparatus (MG160): $r = 0.76$; $P < 0.002$. They were not significant for endoplasmic reticulum ($r = 0.125$; $P = 0.54$) and early endosomes ($r = -0.152$; $P = 0.67$) (Figure 5). This result indicates that the volume of the lysosomes and of the Golgi apparatus was increased in neurons with an increased content of Aβ peptide, while the volume of the endoplasmic reticulum and of the early endosomes did not change. Intracellular Aβ peptide occupied a larger volume in the neurons where a co-localization with cathepsin D was observed than when a co-localization with EEA1 or GRP78 was present (analysis of variance, PLSD $P < 0.01$ in both cases).

Antibodies directed against cathepsin D (Figure 4J), MG 160 (Figure 4D) and GRP78 (Figure 4A) labeled their respective compartment and some Aβ-positive granules. In contrast, the antibody against EEA1 revealed small vesicles, which were, for most of them, devoid of Aβ labeling (Figure 4G) and did not show the granules. Flotillin-1 antibody labeled only some Aβ-positive granules (Figure 4, D to F). SNAP 25 (a synaptic marker) and Cox2 (a marker of mitochondria) did not co-localize with Aβ peptide (not shown).

Immunoelectron Microscopy

At the ultrastructural level, antibodies against $A\beta_{8-17}$ or $A\beta_{17-31}$[46] decorated both intracellular structures and extracellular deposits (Figure 6, B, F and H). A few gold particles were found in the neuronal ER, in the Golgi apparatus, in pre- and post-synaptic structures. Many gold particles decorated intraneuronal multivesicular bodies (MVB). These MVB were ovoid (~500 to 1000 nm small axis, ~700 to 1500 nm long axis) or round structures (~700 to 1000 nm in diameter), limited by a single unit membrane and containing between 10 and 30 intralumenal vesicles and a few dense bodies (Figure 6, A and B). Some vesicles appeared electrolucent, probably because liposoluble material had been extracted through the processing of the sample (Figure 6, A, B, and E). Some other MVB had a dark matrix reminiscent of the lysosomal content (= dark MVB) (Figure 6, C, D, and E).

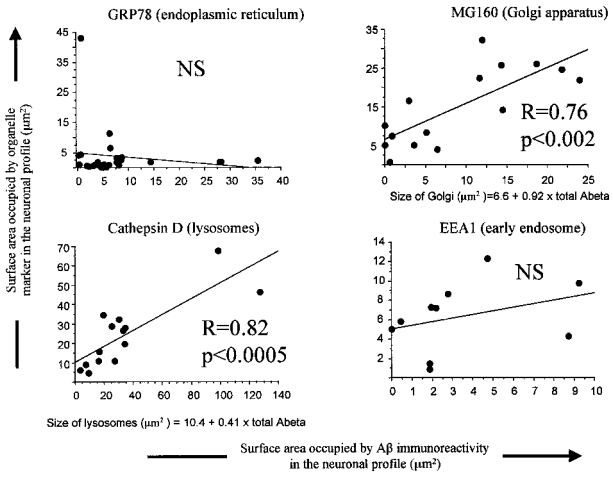

Figure 5. Proportion (%) of the total volume of intracellular Aβ peptide co-localized with the organelle marker. The Aβ antibody is the monoclonal antibody 6FD3, directed against amino acids 8–17 of the peptide (Dako). See Table 2 for details concerning the antibodies.

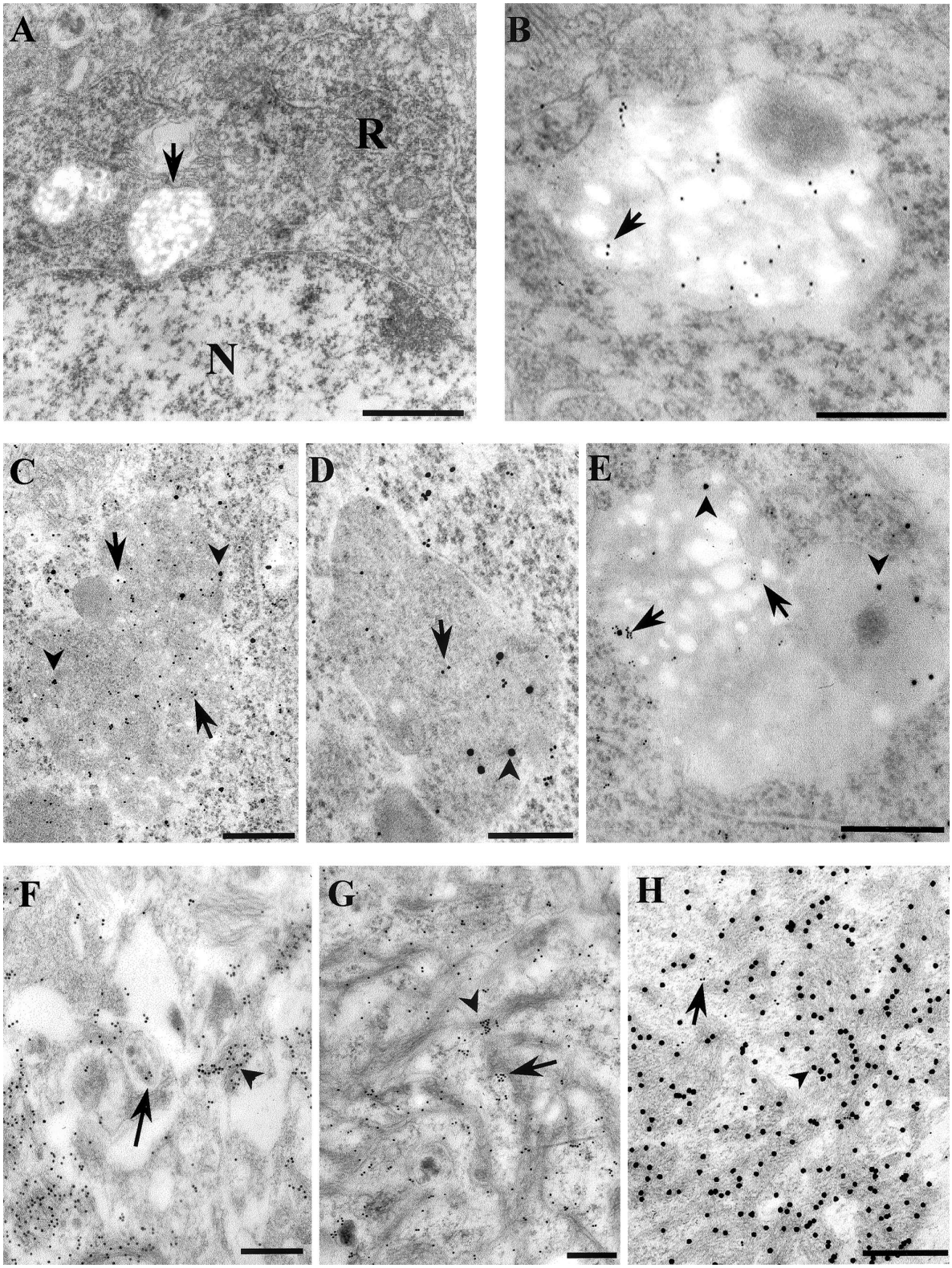

Figure 6. Electron microscopy. **A:** Conventional electron microscopy picture showing the appearance of intracellular granule (**arrow**); N, nucleus; R, endoplasmic reticulum. **B** to **H:** Immunoelectron microscopy. **B:** Labeling of an intraneuronal granule with anti-Aβ_{17-31} (E50,[46]). Gold particles are located in the lumenal vesicles (**arrow**). **C:** Double immunolabeling of intraneuronal granule with anti-Aβ_{17-31} (20-nm gold particle, **arrowhead**) and anti-flotillin (10-nm gold particle, **arrow**). **D:** Double immunolabeling of intraneuronal granule with anti-Aβ_{8-17} (10-nm gold particle, **arrow**) and anti-cathepsin D (20-nm gold particle, **arrowhead**). **E:** Double immunolabeling of a multivesicular body–lysosome with anti-APP Cter (20-nm gold particle, **arrowhead**) and anti-flotillin (10-nm gold particle, **arrow**). **F:** Labeling of an Aβ deposit with the anti-Aβ_{17-31} antibody decorating amyloid fibrils (**arrowhead**). The content of an extracellular vesicle is also labeled (**arrow**). (**G**) Labeling of Aβ deposit with the anti-cathepsin D antibody showing gold particles associated with amyloid fibrils (**arrowhead**) or between them (**arrow**). **H:** Double immunolabeling of an Aβ deposit with anti-Aβ_{17-31} (20-nm gold particle, **arrowhead**) and anti-flotillin (10-nm gold particle, **arrow**). Note that 10-nm gold particles are located in between the typical amyloid fibrils. **Bars A** to **C** and **E** to **H**, 400 nm; **D**, 250 nm.

No MVB contained amyloid fibrils. The Aβ containing MVB were most often located in the perinuclear region (Figure 6A). Double immunoelectron microscopy using two-sized gold particles (10 and 20 nm) showed the co-occurrence of Aβ and flotillin, of Aβ and cathepsin D, of flotillin and APPcter, (Figure 6, C to E) in dark multivesicular bodies.

Aβ-positive extracellular deposits appeared as bundles of 10 nm fibrils, located close to cell processes containing numerous dense bodies or lamellar structures which remained unlabeled. Small vesicles (20 to 60 nm in diameter), containing $A\beta_{17-31,}$ were found admixed with amyloid fibrils within the deposits (Figure 6F). Cathepsin D immunoreactivity (Figure 6G) was observed on or between amyloid fibrils.

Discussion

We have shown that intraneuronal accumulation of Aβ peptide took place in granules visible at light microscopy in Thy-1 APPxPS1 transgenic mice. These granules were detected in the isocortex and hippocampus at 2 months of age, at a stage where Aβ extracellular deposits were not yet present. Their number increased between 2 and 11 months of age (between 2 and 9 months in the hippocampus and brainstem) and decreased thereafter, confirming previous data.[34,36,47] Aβ-positive granules has also been detected in single APP transgenic mice.[36]

In contrast to granules, the number of extracellular Aβ deposits progressed even in aged animals. All of the regions where neurons harbored granules (isocortex, hippocampus, thalamus, and brainstem) contained Aβ deposits from 5 months on. It should be stressed here that the distribution of plaques in these Thy-1 APPxPS1 transgenic mice differs from what is observed in human (thalamic plaques being rare in man). Conversely, neurons were devoid of granules in the regions which did not contain extracellular Aβ deposits (eg, cerebellum and striatum).

At electron miscroscopy, Aβ immunoreactivity was concentrated in MVB whose external diameter corresponded to the mean diameter of granules seen at light microscopy. The lumenal vesicles (20 to 40 nm in diameter) are below the resolving power of the light microscope. Some of these MVB were dark. Their matrix appeared to have the same aspect as the lysosomal matrix, suggesting that fusion with lysosome(s) had occurred.

The Aβ-positive granules reacted with $A\beta_{1-8}$, $A\beta_{8-17}$, $A\beta_{33-40}$, and $A\beta_{35-42}$ antibodies. The labeling of the granules by antibody $A\beta_{1-8}$ and $A\beta_{8-17}$ indicates that the reactive epitope was not a product of the α-cleavage, which involves aa 16 and 17. The labeling by $A\beta_{33-40}$ and $A\beta_{35-42}$ antibodies shows that the reactive sequence was not embedded in APP, since these antibodies recognize an epitope that includes the terminal COOH group.[40] On the other hand, the labeling of the granules by the two C-terminal APP antibodies cannot be interpreted as a cross-reaction with Aβ peptide since these antibodies were raised against peptides that did not include the Aβ sequence. Finally, the absence of labeling of the granules by the APP_{66-81} (22C11 monoclonal) demonstrates that the N-terminus of the protein is excluded from the granules. One may conclude that the granules do not contain full-length APP.

Full-length Aβ peptide and APP C-terminus were close enough to appear co-localized at confocal microscopy (ie, at a distance <250 nm[48,49]). Immunoelectron microscopy confirmed the presence of the C-terminal epitopes of APP in the granules. Only the APP cleaved by the β-secretase (βAPP Cter) appeared to be targeted to the granules. The presence of β-cleaved C-terminal fragments in vesicular compartments was also mentioned in cells overexpressing Rab5.[22] It raises the possibility that γ-secretase cleaves βAPP-Cter within the granules themselves, in agreement with recent findings indicating that PS1 and nicastrin, two constituents of the γ-secretase complex,[50] are localized in the membrane of the lysosome.[26]

Using double immunofluorescence with confocal microscopy, we found that most of the granules were labeled by antibodies directed against constituents of the lysosomes (cathepsin D or LAMP2). Aβ epitopes were sometimes co-localized with MG160 (a marker of Golgi apparatus) and rarely with Grp78/Bip (a marker of the ER). Co-localization of Aβ peptide and cathepsin D was significantly associated with an increased volume of cathepsin D immunoreactivity, suggesting that Aβ peptide produced in excess accumulated in lysosomes or in MVB containing lysosomal enzymes. Of notice in this regard is the presence of LAMPs in the membrane of MVB in cells where it has been studied such as the human neutrophils.[51] Although not as marked, there was also an increase in the volume of the Golgi apparatus while both the ER and early endosomes were of normal size. The normal size of the endosomes raises questions. In sporadic Alzheimer disease, trisomy 21, and in the segmental trisomy 16 mouse model, the volume of the early endosomes is increased,[18,52,53] but it has been found normal in human cases of PS1 mutation and in another line of APPxPS1 double transgenic mice,[53] a normal morphology being obviously still compatible with an increased turnover of APP. As previously shown,[53] overexpression of APP is not sufficient alone to increase the volume of the endosomal compartment and some other factor(s) (located on the chromosome 16 in the trisomy 16 mouse model) seem(s) also necessary. Alternatively, the PS1 mutation (as in our mice or in the human disease[53]) could affect the cellular topography of Aβ production, so preventing the enlargement of the endosomes.

The finding that flotillin-1 antibody (a raft marker[54]) labeled some of the granules and that, ultrastructurally, there was co-occurrence of flotillin, Aβ, and APP Cter in small aggregates found in the MVB, may be of importance in view of the literature indicating that a significant fraction of APP is concentrated in rafts[55,56] and that raft proteins are concentrated in the vesicles of MVB and exosomes.[57] Multivesicular bodies are involved in recycling and degradation of membrane proteins. In several cell types, such as the intestinal cells,[58] the dendritic

cells[59] or B-lymphocytes,[60] the content of MVB are extruded as "exosomes." With immunoelectron microscopy using an anti-Aβ antibody, we showed the presence of gold particles in multivesicular bodies in transgenic mice. Direct evidence of a γ-secretase activity or of the presence of its constituents in multivesicular bodies is still lacking in the nervous system but PS1 has been identified in azurophil granules of polymorphonuclear cells and in α-granules of platelets.[61] Both organelles are considered to be specialized MVB.[32]

The lumenal vesicles of the MVB are known to be enriched in lipids, including cholesterol.[62] This lipid environment could explain why Aβ peptide, whose amino acid sequence is, for a third of its length, hydrophobic, does not aggregate in the hydrophilic cytoplasmic environment.

Our observations provide additional support to previous findings showing that Aβ accumulates in neurons.[7,8,11,12,34,36,47,63] In the present model there is no evidence that intracellular Aβ is rapidly toxic to the neuron: at 2 months, when only granules are present, there is no sign of inflammation, neuritic dystrophy or neuronal loss. The granules could however play a role in the long run, impeding normal cellular function and causing neuronal death.[37]

The origin of the extracellular Aβ has not been fully elucidated. Could it originate from the multivesicular body where it accumulates? In man, as in transgenic mice, the abundance of Aβ-positive granules decreases in the later stages of the disease, while the peptide accumulates in the extracellular space. Moreover, in the transgenic mice, extracellular Aβ deposits and intraneuronal granules occur in the same areas of the brain. These observations are compatible with the initial accumulation of Aβ within the neuron and its secondary secretion outside the cell. Extracellular Aβ was found colocalized with cathepsin D in these transgenic mice as in man.[64,65] The presence of cathepsin D immunoreactivity and enzymatic activity in postmortem CSF of Alzheimer disease patient[66] is also an evidence of its accumulation in the extracellular space. This could be explained by the exocytosis of the MVB-lysosomal content, including both Aβ peptide and cathepsin D. Lysosomes with a secretory activity (so-called "secretory lysosomes") have been described in other systems.[67] They share characteristics of, or are identical with, MVB. Since extracellular Aβ is colocalized with cathepsin D[65], a lysosomal marker, we raise the possibility that Aβ peptide is stored in secretory lysosomes, known, in other cell types, to liberate their content outside the cell.

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