

Alzheimer's disease β -amyloid peptides are released in association with exosomes

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Although the exact etiology of Alzheimer's disease (AD) is a topic of debate, the consensus is that the accumulation of β -amyloid ($A\beta$) peptides in the senile plaques is one of the hallmarks of the progression of the disease. The $A\beta$ peptide is formed by the amyloidogenic cleavage of the amyloid precursor protein (APP) by β - and γ -secretases. The endocytic system has been implicated in the cleavages leading to the formation of $A\beta$. However, the identity of the intracellular compartment where the amyloidogenic secretases cleave and the mechanism by which the intracellularly generated $A\beta$ is released into the extracellular milieu are not clear. Here, we show that β -cleavage occurs in early endosomes followed by routing of $A\beta$ to multivesicular bodies (MVBs) in HeLa and N2a cells. Subsequently, a minute fraction of $A\beta$ peptides can be secreted from the cells in association with exosomes, intraluminal vesicles of MVBs that are released into the extracellular space as a result of fusion of MVBs with the plasma membrane. Exosomal proteins were found to accumulate in the plaques of AD patient brains, suggesting a role in the pathogenesis of AD.

multivesicular bodies | rafts | amyloid precursor protein | β -secretase | endocytosis

Alzheimer's disease (AD) is a late-onset neurological disorder with progressive loss of memory and cognitive abilities as a result of excessive neurodegeneration (1). AD is characterized by extracellular aggregates of β -amyloid ($A\beta$) peptides known as amyloid plaques (2). The $A\beta$ peptide is derived from the sequential processing of the amyloid precursor protein (APP) by β - and γ -secretases. β -secretase [$(\beta$ -APP cleaving enzyme (BACE)] is a type-1 transmembrane aspartyl protease and is mainly localized to endosomes, lysosomes and the trans-Golgi network (3). γ -Secretase is a multicomponent complex that is composed of presenilin-1/presenilin-2, nicastrin, Aph-1, and PEN-2 (4) and is localized to the early secretory (5, 6) and the endocytic compartments (7, 8). Nonamyloidogenic processing of APP involves α -secretase that cleaves APP inside the $A\beta$ region, giving rise to the α -cleaved ectodomain, thus precluding the formation of $A\beta$ (9). Hence, the availability of APP to either α - or β -secretase determines whether $A\beta$ peptide will be generated. Lateral organization of membranes (10) and subcellular localization (11, 12) of the substrate and the secretases have been documented to regulate $A\beta$ generation. Recent work suggests that β -secretase associates with lipid rafts, liquid-ordered domains in the membrane (13, 14), and that integrity of raft domains is required for β -cleavage of APP to occur (ref. 10; see, however, ref. 15). α -Cleavage, in contrast, occurs outside raft domains (10). The γ -secretase complex is also raft-associated (16); hence, amyloidogenic processing of APP could occur in clustered raft domains to generate $A\beta$ (10). Inhibition of endocytosis reduces β -cleavage but not α -cleavage, suggesting that β -cleavage mainly occurs in endosomes (10, 11, 17–19). Accumulation of $A\beta$ peptides in extracellular plaques requires the release of $A\beta$ peptides from the cell. An intriguing question is how the intracellularly generated, fairly hydrophobic $A\beta$ peptide is released into the extracellular space. Here we show that

β -cleavage occurs in a specific subset of endosomes and that a fraction of $A\beta$ peptides is found in multivesicular bodies (MVBs) and is released in association with exosomes. Upon fusion of MVBs with the plasma membrane, the intraluminal vesicles of MVBs are released into the extracellular milieu as exosomes. These vesicles are enriched in raft lipids and proteins and are implicated in various functions, such as scavenging of archaic proteins, signaling, and transmission of pathogens (20). In this study, we also show that an exosome-associated protein, Alix, is specifically enriched in amyloid plaques of AD brain sections, suggesting a novel role for exosomes in AD pathogenesis.

Results

β -Cleavage of APP Occurs in Early Endosomes. To identify the intracellular compartment within the endocytic pathway where β -cleavage occurs, we performed immunofluorescence experiments in HeLa cells expressing the Swedish mutant of APP (swAPP) (2) by using an antibody that specifically recognizes the β -cleaved ectodomain of swAPP (sAPP β) (Fig. 6, which is published as supporting information on the PNAS web site) along with markers for different endosome populations (Fig. 1*b*). Colocalization of sAPP β was observed with the early endosomal markers, rab5 (47% of colocalization) and early endosomal antigen-1 (EEA-1), suggesting early endosome to be a station of β -cleavage (Fig. 1*a* and *b*). Colocalization of sAPP β was also observed with the late endosomal marker, rab7 (58% of colocalization), but little colocalization was observed with rab11 (29% of colocalization) (Fig. 1*b*) or with transferrin, 20 min after internalization (data not shown). Similar results were also obtained with the neuroblastoma cells, N2a (Fig. 7, which is published as supporting information on the PNAS web site).

Because the β -cleaved ectodomain was found both in early and late endosomes under these steady-state conditions, it is possible that β -cleavage either occurs in early endosomes, and the cleaved ectodomain is then transported to late endosomes or that the cleavage occurs both in early and late endosomes. By overexpressing the GTPase mutant of rab5 (rab5Q79L), which inhibits cargo flow from early to late endosomes (21), we could freeze almost all of the cellular β -cleaved ectodomain in these enlarged endosomes consistent with the idea that β -cleavage occurs either in or upstream of early endosomes (Fig. 1*b*). Overexpression of this dominant active version of rab5, however, neither enhanced β -cleavage nor increased the cellular levels of β -cleaved ectodomain (Fig. 8, which is published as supporting information on the PNAS web site), suggesting that the secretion of sAPP β was not affected under these conditions. The involvement of early endosomes in β -cleavage was confirmed by induced endocytosis experiments in which APP and BACE were crosslinked with

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Abbreviations: $A\beta$, β -amyloid; AD, Alzheimer's disease; APP, amyloid precursor protein; swAPP, Swedish APP; sAPP β , β -cleaved ectodomain of swAPP; BACE, $A\beta$ cleaving enzyme; MVBs, multivesicular bodies; CFP, cyan fluorescent protein; MSD, Meso Scale Discovery.

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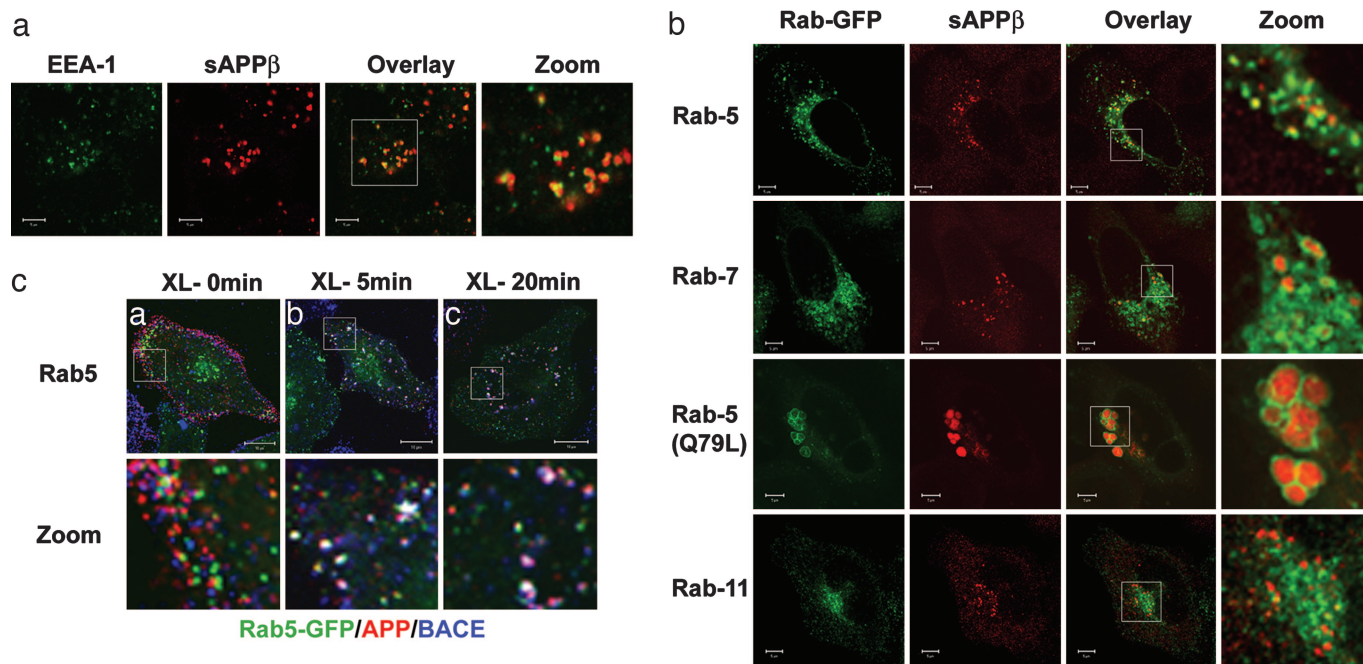


Fig. 1. Involvement of early endosomes in β -cleavage of APP. (a) HeLa cells expressing swAPP were stained for endogenous early endosomal antigen-1 (EEA-1) (green) and the β -cleaved ectodomain (sAPP β) (red). (b) HeLa cells expressing swAPP were transfected with rab5, rab7, dominant active rab5 mutant (Q79L), or rab11 GFP fusion proteins fixed and stained for the β -cleaved ectodomain (sAPP β) (red) with ANJJ antibody. (c) HeLa cells expressing the rabGFP constructs, APP, and BACE were crosslinked at 4°C with anti-APP (red) and anti-BACE (blue) antibodies and endocytosed at 37°C. After 5 min, APP and BACE are internalized to rab5-GFP-positive endosomes. For a detailed version, see Fig. 9.

antibodies at the cell surface and internalized. After 5 min of internalization, APP and BACE colocalized in rab5-positive early endosomes (Fig. 1c; see also Fig. 9, which is published as supporting information on the PNAS web site). Fluorescence resonance energy transfer measurements also confirmed that maximum intermolecular interaction between APP and BACE occurred in early endosomes [our unpublished results and ref. 22). Interestingly, expression of the dominant negative mutant of rab7 did not affect β -cleavage and A β secretion (data not shown), but expression of the dominant negative mutant of dynamin inhibited β -cleavage and A β secretion by \approx 60%. The absence of a complete inhibition in β -cleavage of swAPP under endocytosis-inhibited conditions could be explained by the observations that swAPP also could undergo β -cleavage during its biosynthetic transport (12, 23). Stimulation of the recycling of cargo from the early endosome back to the plasma membrane by overexpression of wild-type rab4 (24) decreased β -cleavage and A β secretion (Fig. 2), showing that reducing the residence time of APP and/or BACE in early endosomes reduces β -cleavage, suggesting early endosomes to be a major site of β -cleavage.

A Fraction of A β Peptides Is Localized to MVBs and Is Released in Association with Exosomes. Most of the extracellular A β is known to be soluble (23, 25, 26), but we were interested in knowing whether a fraction of the extracellular A β remained membrane-bound as A β is generated in the membrane (27, 28). Therefore, we performed differential centrifugation of the culture supernatants and analyzed for A β peptides in the pelleted membrane fractions. Indeed, a minute fraction pelleted with membranes (Fig. 10, which is published as supporting information on the PNAS web site). Moreover, immunogold labeling of N2a cells with an anti-A β antibody showed that A β peptides also were localized to MVBs (Fig. 3), consistent with previous reports (29, 30). Proteins that are destined for lysosomal degradation are sorted from the early endosomes to intraluminal vesicles of the MVBs or late endosomes. These multivesicular endosomes can then either fuse with lyso-

somes and degrade the cargo or fuse with the plasma membrane to release these vesicles as exosomes (31).

To test whether A β could be released in association with exosomes, we isolated exosomes from culture supernatants of N2a cells stably transfected with swAPP through differential centrifugation and density gradient centrifugation as described in *Materials and Methods* (32, 33). Alix and flotillin-1 were used as marker proteins (34, 35) to identify exosome-containing fractions (Fig. 4a). These fractions were devoid of nonexosomal proteins, such as transferrin receptor and full-length APP (Fig. 4a). Only A β peptides but none of the other APP-derived

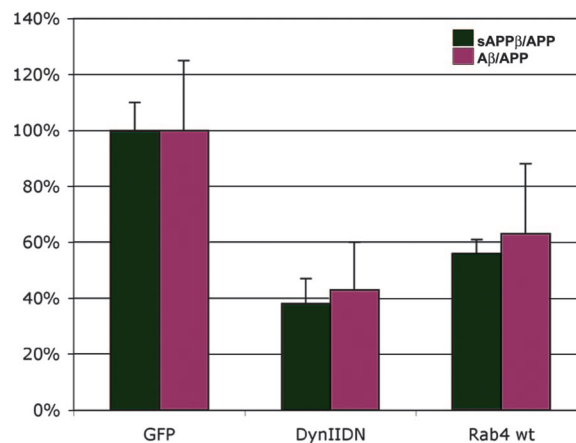
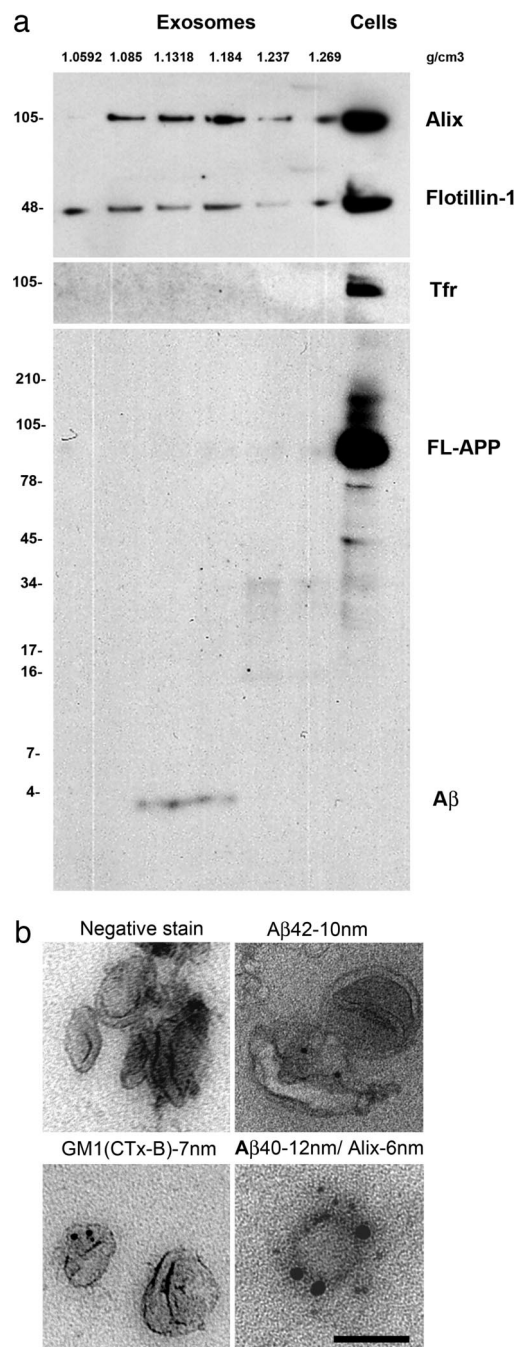


Fig. 2. The effect of endocytic effectors on β -cleavage and A β secretion. N2a cells expressing CFP-swAPP and the dynaminK44A mutant (DynIIDN) or rab4wt constructs were assayed for sAPP β , A β , and full-length APP as described in *Materials and Methods*. The y axis represents the amount of sAPP β as a fraction of full-length APP (sAPP β /APP) (black) or A β as a fraction of full-length APP (A β /APP) (magenta). The GFP control was normalized to 100%.

The data of the present study substantiate that the endocytic pathway is a major site for A β generation. β -cleavage occurs in early



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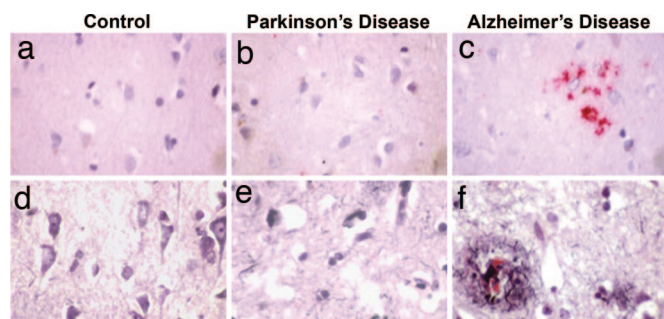


Fig. 5. Exosomal proteins are enriched in human amyloid plaques. Paraffin-embedded human autopsy tissues from patients with AD or Parkinson's disease or from controls were studied. We performed either Gallyas silver stain and subsequent immunohistochemistry for Alix on hippocampal sections (*d–f*) or immunohistochemistry with hematoxylin counterstain only (*a–c*). Alix was stained by indirect alkaline phosphatase method with new fuchsin as the chromogen. (Magnification, $\times 63$.) (*a* and *d*) Hippocampus of a normal control brain without amyloid plaques or detection of Alix. (*b* and *e*) Hippocampus of a patient with Parkinson's disease displaying slight accumulation of pathological tau-filaments but no amyloid plaques and no Alix. (*c* and *f*) Hippocampus of a patient with moderate to advanced Alzheimer's pathology [CERAD (Consortium to Establish a Registry for Alzheimer's Disease) score B] with discrete deposits of Alix within typical neuritic plaques (*f*) containing amyloid, hyperphosphorylated tau-fibrils, and cellular elements (astrocytes and microglia).

endosomes, and γ -cleavage can take place there (38). How the bulk of $A\beta$ is released from its membrane-bound state into the extracellular space is currently not understood. We show here that a minute fraction ($<1\%$) of $A\beta$ is secreted into the extracellular medium bound to exosomes. Because only a minor component of $A\beta$ is associated with exosomes, it raises a concern about whether extracellular soluble $A\beta$ simply “sticks” to the extracellular exosomes or a small fraction of $A\beta$ peptides are indeed released via exosomes. Several observations support the idea that some of the $A\beta$ is released via exosomes: (*i*) $A\beta$ peptides are seen in MVBs (Fig. 3), and these MVBs fuse with the plasma membrane and release the intraluminal vesicles as exosomes that bear $A\beta$. (*ii*) After several stringent washes during the cryoelectron microscopy specimen preparation, $A\beta$ was still seen on the exosomes, which supports that $A\beta$ must be inserted in the membrane. However, it is important to note that additional $A\beta$ also could be carried by multivesicular endosomes and be released in a non-membrane-bound form after fusion with the plasma membrane. Initial reports on characterization of extracellular $A\beta$ used immunoprecipitation and Western blotting analysis and, hence, did not identify the membrane bound, extracellular $A\beta$ (26). Amyloidogenic processing of APP also occurs in the biosynthetic route, and this pool of $A\beta$ could be a component of the nonexosomal release (5). Macroautophagy, a process by which both the biosynthetic and endocytic organelles are degraded, has been recently shown to be involved in $A\beta$ generation (39). $A\beta$ generated by this process also could contribute to the exosomal fraction of $A\beta$ via fusion with late endosomes (39).

Whether exosome-associated $A\beta$ could play a role in the pathogenesis of AD is yet to be studied. The observations that exosome-associated proteins, such as Alix and flotillins (data not shown and ref. 37), are enriched in the amyloid plaques suggest that exosome-associated $A\beta$ may be involved in plaque formation. We have recently shown that raft lipids (neutral sphingolipids and cholesterol) activate β -secretase in proteoliposomes (40). Because exosomes are enriched in glycolipids, raft lipids, and raft-associated proteins, such as glycosylphosphatidylinositol-anchored proteins (34, 35), raft-bound $A\beta$ could be incorporated into exosomes. $A\beta$ -associated GM1, a ganglioside, has been shown to act as an amyloid seed for $A\beta$ fibrillation (41), and the presence of GM1 and other raft lipids (42) in exosomes

bolsters the idea that exosomes could act as nucleation centers for plaque formation. Another amyloid fibril-forming protein, Pmel1, involved in melanosome biogenesis and melanin polymerization (43) has been shown to be routed to MVBs and to be released via exosomes (44). These studies hypothesized that the intraluminal vesicles of MVBs or exosomes could provide a conducive environment for amyloidogenic fibril formation (43). Because AD is a slow and progressive disorder, the release of $A\beta$ associated with exosomes, although minute, could still be involved in the progression of the disease. $A\beta$ could also hitch on exosomes to mediate its intercellular transfer (45). HIV uses the exosome pathway for its assembly and release (46), and prion proteins mediate their intercellular transfer via exosomes (35). Exosomes are thus emerging as Trojan horses in pathogenesis. Further work will be required to assess the significance of the exosome route for the extracellular release of both soluble and membrane-bound $A\beta$.

Materials and Methods

Constructs and Generation of Recombinant Adenoviruses. The cyan fluorescent protein (CFP)-swAPP construct was similar to the yellow fluorescent protein-APP construct described previously (10). The BACE1A-vesicular stomatitis virus glycoprotein construct also was described previously (10). Plasmids encoding rab proteins fused to GFP were obtained from Marino Zerial (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany).

Cells. Mouse neuroblastoma N2a cells and HeLa cells were cultured at 37°C in DMEM supplemented with 10% FCS/100 units/ml penicillin/100 $\mu\text{g}/\text{ml}$ streptomycin. Gang Yu (University of Texas Southwestern Medical Center, Dallas) kindly provided HeLa cells stably expressing swAPP. N2a cells stably expressing the wild-type APP (N2a-wtAPP) and the Swedish mutant (N2a-swAPP) were kind gifts from Gopal Thinakaran (University of Chicago, Chicago) and were cultured as described in ref. 23. Adenovirus expressing either CFP-swAPP or swAPP-CFP and BACE-vesicular stomatitis virus glycoprotein were used to infect both N2a and HeLa cells. In some cases, plasmids encoding for CFP-swAPP or swAPP-CFP were also used.

Antibodies. Rabbit polyclonal antibody 7523 against the N-terminal end of BACE1 was a kind gift from Christian Haass (Ludwig-Maximilians-University, Munich, Germany) (47). Mouse monoclonal antibodies against Alix/AIP1, Flotillin-2, Flotillin-1, and Transferrin receptor were from BD Biosciences (Heidelberg, Germany). The fluorophore-conjugated (Cy2, Cy3, Cy5, or FITC) secondary antibodies were purchased from The Jackson Laboratory. Monoclonal antibodies against A β 40 and A β 42 (G2-10 and G2-13, respectively) were from The Genetics Company (Zurich, Switzerland). Monoclonal antibodies 6E10, which recognizes the 1–12 region of $A\beta$ peptide, and 4G8, which is directed against the 17–24 region of $A\beta$, were purchased from Biocat (Heidelberg, Germany). Rabbit polyclonal antibodies IP60 (10), which is directed against the C terminus of APP, 70JE (10), which is directed against amino acids 1–11 of $A\beta$, and ANJJ, which is directed against the C terminus of the sAPP β , were developed in our laboratory. ANJJ recognizes sAPP β of swAPP but no other APP-processing products.

Reagents. Alexa Fluor 546-conjugated Transferrin and the unconjugated holotransferrin were purchased from Molecular Probes. Cholera toxin B and other routinely used chemicals were purchased from Sigma. All reagents for the Meso Scale Discovery (MSD) electrochemiluminescence assays were purchased from Meso Scale Discovery (Gaithersburg, MD).

Transfection, Viral Infection, and Biochemical Assays. At 24 h after the seeding of N2a cells into 3.5-cm dishes, transient transfection

tions were performed with 1–3 μg of each expression plasmid either using calcium phosphate precipitation as described by Chen and Okayama (48) or Lipofectamine 2000 (Invitrogen). Additionally, at 24 h after seeding, the N2a cells were infected with recombinant adenoviruses for 1 h at 37°C in complete medium. After a change of medium, the cells were incubated for 16–20 h at 37°C and then used for biochemical assays.

Immunoprecipitation and Quantification. After metabolic labeling for 1 h, the cell culture medium was collected, and cell extracts were prepared in PBS containing 2% Nonidet P-40, 0.2% SDS, and chymostatin, leupeptin, antipain, and pepstatin A each at 25 $\mu\text{g}/\text{ml}$. Immunoprecipitates were recovered on protein A-Sepharose CL4B beads (Amersham Pharmacia Biosciences) preincubated with antibodies IP60, ANJJ, and 70JE, respectively, and separated on 10–20% Tris-tricine (Invitrogen) gels. PhosphorImager plates were exposed to the fixed and dried gels, and bands were quantified by using the Fuji BAS 1800II image plate reader and Science Lab 99 IMAGE GAUGE 3.3 software (Raytest Isotopenmessgeraete, Straubenhardt, Germany).

Immunofluorescence Microscopy. Coverslip-grown cells were transfected with various rab-GFP constructs and infected with adenoviruses expressing the swAPP construct. In the case of N2a cells, cells were grown on polylysine/laminin-coated coverslips. After 6–8 h of transfection/infection, the cells were fixed with 3.7% paraformaldehyde, washed with ammonium chloride, permeabilized with methanol at 20°C 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 in blocking buffer for 1 h, subsequently washed thoroughly with PBS, and the primary signal was detected with various fluorochrome-conjugated (FITC, Cy2, Cy3, or Cy5) anti-mouse or anti-rabbit antibodies.

Antibody Crosslinking and Induced Endocytosis. Coverslip-grown cells were transfected with either rab-GFP or control GFP constructs and infected with adenoviruses expressing swAPP and BACE. After 6–8 h of transfection/infection, cells were washed twice in CO_2 -independent medium containing 2% BSA (BCM). Cells were then incubated in the cold (4°C) with anti-APP (6E10) and anti-BACE (7523) in BCM for 1 h and then washed and further incubated with donkey anti-mouse Cy3 and donkey anti-rabbit Cy5 antibodies in BCM. The cells were then washed and incubated at 37°C for 5, 10, and 20 min. After these times, the cells were immediately fixed in paraformaldehyde and processed as described in *Immunofluorescence Microscopy*.

Preparation of Exosome-Depleted Medium. The medium was depleted of exosomes from bovine serum by essentially following an established protocol (35). DMEM/glutamine containing 20% FCS and penicillin/streptomycin (Invitrogen) was centrifuged overnight at 4°C and 100,000 $\times g$ using a Ti45 rotor in a Beckmann ultracentrifuge. The supernatant was carefully removed with a pipette and passed through a vacuum-connected 0.22- μm filter. This medium was then added to an equal volume of DMEM/glutamine containing penicillin/streptomycin and used for the purification of exosomes as described below.

Purification of Exosomes. Exosomes from untransfected or APP stably transfected N2a cells were prepared as described (35). Briefly, cells from 40–60 T175 flasks were cultured in DMEM with 10% FCS. A day before the exosome preparation, culture medium was replaced with exosome-depleted medium. Culture supernatants of cells grown for 24 h were collected and spun at 300 $\times g$ for 10 min to remove cells. The supernatants were then sequentially centrifuged at 1000 $\times g$, 10,000 $\times g$, and 100,000 $\times g$.

The 100,000 $\times g$ pellet was resuspended in 2.5 M sucrose in 20 mM Hepes (pH 7.4), and a step gradient of sucrose (2.25, 2.0, 1.75, 1.5, 1.25, 1.0, 0.75, 0.5, and 0.25 M) was layered over the exosome-containing, 2.5 M sucrose solution. The gradient was spun at 200,000 $\times g$ for at least 16 h using an SW55 rotor. Fractions were collected from the top of the gradient, diluted with PBS, and spun at 100,000 $\times g$ with a TLA-100.3 rotor. The pelleted fractions were then used either for immunoblotting or for electron microscopy.

Immunoelectron Microscopy and MSD Electrochemiluminescence Assays. The isolated exosome fractions (fractions 3 and 4 from the gradient) and the cells were immunolabeled as described in refs. 35 and 49. MSD assays for A β 1–40, sAPP α , sAPP β , and full-length APP were performed on a SECTOR Imager 6000 (Meso Scale Discovery). This platform is based on MSD's MultiArray technology, which is a proprietary combination of patterned arrays and electrochemiluminescence detection enabling large numbers of measurements with exceptional sensitivity, wide dynamic range, and convenience. Individual wells of a High-Bind MultiArray 384-well-plate were coated with capture antibodies for A β 1–40 (6E10), sAPP α (6E10), sAPP β (ANJJ), and full-length APP (IP60). After a blocking step with 3% MSD Blocker A in MSD Tris wash buffer, 25- μl aliquots of the cell lysate, the 100,000 $\times g$ supernatant, and the 100,000 $\times g$ pellet were added to individual wells for 1 h at room temperature with shaking. After four washes with 40 μl of MSD Tris wash buffer, the SULFO-TAG-labeled detection antibodies were added in 25 μl of 1% MSD Blocker A in MSD Tris wash buffer. They were prepared by incubating antibody G2–10 (for A β 1–40) or an antibody binding close to the N terminus of APP (for sAPP α , sAPP β , and full-length APP) with SULFO-TAG-NHS-ester [ruthenium(II) tris-bipyridine, *N*-hydroxysuccinimide; Meso Scale Discovery] as suggested by the manufacturer. Note that the latter antibody binds human, but not mouse, APP. After an incubation for 1 h at room temperature with shaking, the wells were washed again, 35 μl of MSD read buffer T with surfactant was added, and the plates were read immediately.

Histology and Immunocytochemistry on Human Autopsy Tissues. Sections (5 μm thick) of formalin-fixed and paraffin-embedded autopsy tissues of the left hippocampus (obtained from the Department of Pathology, University Clinic, Technical University, Dresden) were deparaffinized and then received Gallyas silver stain (reagents from Merck, Darmstadt, Germany) (50) and subsequent immunocytochemistry for Alix or immunocytochemistry only. The slides were first heated 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 with the use of the ABC indirect alkaline phosphatase kit mouse IgG (Vector Laboratories, Burlingame, CA) and a fuchsin derivative (new fuchsin; Sigma) as a chromogen (51).

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