

# The Exosome Secretory Pathway Transports Amyloid Precursor Protein Carboxyl-terminal Fragments from the Cell into the Brain Extracellular Space\*

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**Background:** Exosomes isolated *in vitro* contain full-length amyloid- $\beta$  precursor protein (flAPP) and APP metabolites.

**Results:** Exosomes secreted *in vivo* in brains of wild-type and APP-overexpressing mice contain higher levels of APP C-terminal fragments (CTFs) relative to flAPP compared with brain tissue.

**Conclusion:** Brain exosomes are enriched with APP CTFs.

**Significance:** The exosome secretory pathway clears cellular APP CTFs, releasing the toxic fragments into the neuropil.

*In vitro* studies have shown that neuronal cell cultures secrete exosomes containing amyloid- $\beta$  precursor protein (APP) and the APP-processing products, C-terminal fragments (CTFs) and amyloid- $\beta$  (A $\beta$ ). We investigated the secretion of full-length APP (flAPP) and APP CTFs via the exosome secretory pathway *in vivo*. To this end, we developed a novel protocol designed to isolate exosomes secreted into mouse brain extracellular space. Exosomes with typical morphology were isolated from freshly removed mouse brains and from frozen mouse and human brain tissues, demonstrating that exosomes can be isolated from post-mortem tissue frozen for long periods of time. flAPP, APP CTFs, and enzymes that cleave both flAPP and APP CTFs were identified in brain exosomes. Although higher levels of both flAPP and APP CTFs were observed in exosomes isolated from the brains of transgenic mice overexpressing human APP (Tg2576) compared with wild-type control mice, there was no difference in the number of secreted brain exosomes. These data indicate that the levels of flAPP and APP CTFs associated with exosomes mirror the cellular levels of flAPP and APP CTFs. Interestingly, exosomes isolated from the brains of both Tg2576 and wild-type mice are enriched with APP CTFs relative to flAPP. Thus, we hypothesize that the exosome secretory pathway plays a pleiotropic role in the brain: exosome secretion is beneficial to the cell, acting as a specific releasing system of neurotoxic APP CTFs and A $\beta$ , but the secretion of exosomes enriched with APP CTFs, neurotoxic proteins that are also a source of secreted A $\beta$ , is harmful to the brain.

bodies (MVBs)<sup>3</sup> around cytoplasmic materials, including proteins and RNAs. Mature exosomes remain inside the lumen of the MVB until they are secreted into the extracellular space when the MVB fuses with the cytoplasmic membrane (1). Secreted exosomes are taken up by target cells, and exosome content is delivered into the recipient cell (2). The composition of the double-layer membrane renders exosomes more stable in the extracellular environment compared with soluble proteins (reviewed in Ref. 3). Exosomes do not contain a random array of intracellular proteins, but a specific set of protein families arising from the plasma membrane, the endocytic pathway, and the cytosol. In addition, exosomes harbor distinct subsets of proteins that are cell type-specific.

Secreted exosomes were first isolated from the conditioned medium of immature sheep reticulocytes (4). Since then, *in vitro* studies have shown that exosomes are secreted by various cell types (3). The presence of exosomes in the nervous system *in vivo* has not been demonstrated, but tissue culture studies have shown that neurons and astrocytes release exosomes (5). Rat and mouse cortical neurons secrete exosomes in culture that have the typical features of size, density, and saponin sensitivity (5). Using proteomic methods, it was shown that these exosomes resemble exosomes isolated from other non-neuronal cell types, containing typical exosomal markers such as alix, flotillin, and TSG101 (tumor susceptibility gene-101) but also containing neuron-specific components (5).

Exosome secretion was originally described as a complementary process to the lysosomal and proteasomal degradative pathways for shedding obsolete membrane and cytosolic proteins in differentiating reticulocytes (4). Identification of neuron-specific components associated with exosomes (5) suggested that secreted exosomes have roles also in cell signaling functions (6), shuttling cargo between cells and tissues (7), regulating neurotransmitter receptor levels at the synapse, controlling the production and turnover of myelin membrane pro-

Exosomes are small membranous vesicles formed by the invagination of the membrane of endosomal multivesicular

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<sup>3</sup> The abbreviations used are: MVB, multivesicular body; A $\beta$ , amyloid- $\beta$ ; AD, Alzheimer disease; flAPP, full-length amyloid- $\beta$  precursor protein; CTF, C-terminal fragment; AChE, acetylcholine esterase; PrP, prion protein.

teins, and participating in the progression of neurodegenerative diseases by relieving the cell from infectious and cytotoxic materials that accumulate in the MVBs (8–10).

A pathogenic function of exosomes was proposed suggesting that it is a pathway for transfer of pathogens between cells. One such pathogen that exploits this pathway is the prion, the infectious particle responsible for transmissible neurodegenerative diseases such as Creutzfeldt-Jakob disease in humans and bovine spongiform encephalopathy in cattle (9). A pathogenic role for exosomes was also proposed for amyloid- $\beta$  (A $\beta$ ) deposited in the brains of Alzheimer disease (AD) patients. Exosomes isolated from the conditioned medium of neuronal cell cultures transport the full-length amyloid- $\beta$  precursor protein (flAPP), APP metabolites, and the enzymes that cleave both flAPP and APP C-terminal fragments (CTFs) to the extracellular space (11). On the basis of these *in vitro* studies, we undertook to determine the effect of higher levels of APP expression in the brains of transgenic mice overexpressing human APP with the K670N/M671L Swedish double mutation (Tg2576) (12) on the number of exosomes secreted and exosomal levels of flAPP and APP CTFs. Although exosomes were isolated from conditioned culture media and bodily fluids, including the cerebrospinal fluid, blood, and urine (13), exosomes secreted into the extracellular space of tissues have not been described. Therefore, we designed a novel protocol to isolate exosomes from either fresh or frozen brain tissue. We show here that in accordance with the high levels of flAPP and APP CTFs in the brains of Tg2576 mice, exosomes secreted into the extracellular space of these mice contain higher levels of flAPP and APP CTFs than exosomes secreted in the brains of wild-type control mice. Interestingly, we demonstrate that the ratio of APP CTFs to flAPP is higher in brain exosomes compared with brain homogenates in both Tg2576 and non-transgenic mice. These data show that the amount of flAPP and APP CTFs secreted out of the cell by brain exosomes is proportional to their brain levels but that brain exosomes are specifically enriched with APP CTFs regardless of levels of APP expression.

## EXPERIMENTAL PROCEDURES

**Mouse Lines and Brain Tissue**—We isolated exosomes from the brains of 16–17-month-old APP transgenic mice (Tg2576) and age- and gender-matched wild-type controls. Either freshly removed or frozen brains were used. In each experiment, exosomes were simultaneously isolated from the brains of a transgenic and a wild-type littermate non-transgenic control mice. For each brain, the right hemi-brain was processed for exosome isolation, and the left hemi-brain was homogenized in cold radioimmune precipitation assay lysis buffer (50 mM Tris-HCl, 1% Nonidet P-40, 150 mM NaCl, and 1 mM EDTA, pH 7.4) supplemented with a mixture of protease inhibitors (P8340, Sigma) for protein analysis by Western blotting. All animal procedures were performed following the National Institutes of Health guidelines with approval from the Institutional Animal Care and Use Committee at the Nathan S. Kline Institute for Psychiatric Research. All efforts have been made to minimize animal suffering and the numbers of mice used.

**Human Brain Tissue**—We isolated exosomes from Brodmann area 9 of human tissue obtained from the Harvard Brain

Tissue Resource Center (Belmont, MA). The tissues were obtained from an AD patient, Braak V (14), a 73-year-old female (post-mortem interval of 17.67 h), and a neuropathologically normal control, a 70-year-old male (post-mortem interval of 25.83 h).

**Exosome Isolation and Purification** (see Fig. 1)—Fresh or previously frozen murine hemi-brains were dissected and treated with 20 units/ml papain (Worthington) in Hibernate E solution (3 ml/hemi-brain; BrainBits, Springfield, IL) for 15 min at 37 °C. The brain tissue was gently homogenized in 2 volumes (6 ml/hemi-brain) of cold Hibernate E solution. The brain homogenate was sequentially filtered through a 40- $\mu$ m mesh filter (BD Biosciences) and a 0.2- $\mu$ m syringe filter (Thermo Scientific). Exosomes were isolated from the filtrate as described previously (15). Briefly, the filtrate was sequentially centrifuged at 300  $\times$  *g* for 10 min at 4 °C, 2000  $\times$  *g* for 10 min at 4 °C, and 10,000  $\times$  *g* for 30 min at 4 °C to discard cells, membranes, and debris. The supernatant was centrifuged at 100,000  $\times$  *g* for 70 min at 4 °C to pellet exosomes. The exosome pellet was resuspended in 60 ml of cold PBS (Invitrogen), and the exosome solution was centrifuged at 100,000  $\times$  *g* for 70 min at 4 °C. The washed exosome pellet was resuspended in 2 ml of 0.95 M sucrose solution and inserted inside a sucrose step gradient column (six 2-ml steps starting from 2.0 M sucrose up to 0.25 M sucrose in 0.35 M increments, with the 0.95 M sucrose step containing the exosomes). The sucrose step gradient was centrifuged at 200,000  $\times$  *g* for 16 h at 4 °C. One-ml fractions were collected from the top of the gradient, and fractions flanking the interphase separating two neighboring sucrose layers were pooled together for a total of seven fractions (a, top 1-ml fraction; b, 2-ml; c, 2-ml; d, 2-ml; e, 2-ml; f, 2-ml; and g, bottom 1-ml fraction). These fractions were diluted in cold PBS and centrifuged at 100,000  $\times$  *g* at 4 °C for 70 min. Sucrose gradient fraction pellets were resuspended in 20  $\mu$ l of cold PBS. Two  $\mu$ l were used to measure acetylcholine esterase (AChE) activity, and 2- $\mu$ l were used for EM. Exosome lysate was prepared by mixing 16  $\mu$ l of the leftover solution with an equal volume of 2 $\times$  radioimmune precipitation assay lysis buffer supplemented with a mixture of protease inhibitors. We used 2  $\mu$ l of the lysate to quantify exosomal protein content (BCA protein assay kit, Pierce) and 10  $\mu$ l of the lysate (31% of the exosome lysate total volume) for protein analysis by Western blotting.

**AChE Activity Assay**—The AChE activity assay was based on the Ellman assay described previously (16). Briefly, 2  $\mu$ l of exosome resuspended in PBS were diluted in 298  $\mu$ l of AChE assay working solution (1.25 mM acetylthiocholine (A5751, Sigma) and 0.1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (D8130, Sigma) in 0.1 M phosphate buffer at pH 8.0) and incubated at 37 °C in the dark for 30 min. Absorbance was measured at 412 nm to quantify AChE activity in the exosome solution.

**Western Blot Analysis**—Brain homogenates (10  $\mu$ g of protein) and exosomal proteins (10  $\mu$ l of the lysate corresponding to 31% of the exosome lysate total volume) were separated by 4–20% Tris/glycine gel electrophoresis (Criterion precast gel, Bio-Rad). The proteins were electrophoretically transferred onto PVDF membranes (Immobilon, Millipore), and the membranes were incubated with the following antibodies: anti-flotillin (1:1000; BD Transduction Laboratories), anti-APP (C1/

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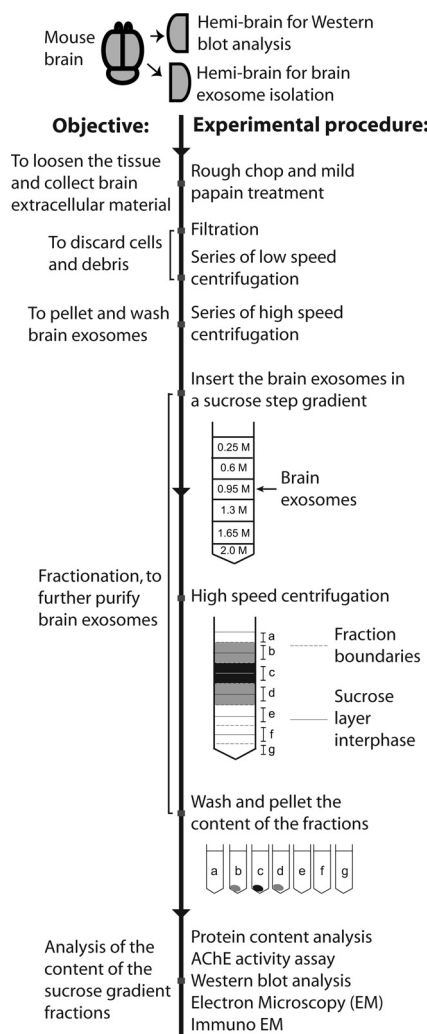
6.1, 1:1000) (17), 6E10 (1:1000; Signet, Princeton, NJ), anti-ADAM10 (1:2000; Millipore), anti-BACE1 (1:1000; Millipore), and anti-nicastrin (1:1000; Millipore). Protein bands were quantified using NIH ImageJ.

**EM and Immuno-EM**—Two  $\mu\text{l}$  of exosome solutions were fixed in 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS. Fractions collected from the sucrose gradient column were subjected to analysis by EM. Immuno-EM analysis of these fractions does not work due to residual sucrose in the samples. Aliquots of the exosome solution, retrieved prior to purification on a sucrose step gradient column, were immunostained as described previously (15) with antibodies to TSG101 (Abcam, Cambridge, MA); flotillin (BD Biosciences); flAPP and APP CTFs (C1/6.1); flAPP, APP CTFs, and A $\beta$  (4G8, Covance Antibody Services, Dedham, MA); and flAPP, APP CTFs, and A $\beta$  (6E10, Signet). Images were captured on a Hamamatsu digital camera (model C4742-95) attached to a Philips CM10 electron microscope using Advantage CCD camera system software (Advanced Microscopy Techniques Corp.).

**Statistical Analyses**—Student's *t* test and analysis of variance were performed using SPSS 15.0 (IBM, Armonk, NY). All results are the combination of data collected from at least three independent experiments.

## RESULTS

**Fresh and Frozen Mouse Brain Tissues Yield Intact Secreted Exosomes**—We have developed a protocol designed to isolate exosomes from the extracellular space of freshly removed murine brain tissue (Fig. 1). Exosomes were isolated from one-hemi-brain of each mouse, and the other hemi-brain was homogenized for Western blot analysis. Murine hemi-brains were treated with papain in Hibernate E solution to loosen the hemi-brain extracellular matrix and to release extracellular material, including brain exosomes in solution. This procedure is widely used for harvesting viable primary neurons from fresh brain tissue (18). The mild papain treatment does not trigger cell lysis and thus prevents the contamination of the extracellular fluid with intracellular organelles and vesicles. Filtration of the brain extracellular material is performed to discard brain cells and debris, followed by a series of centrifugation steps to isolate brain exosomes (15). Brain exosomes were further purified by fractionation on a sucrose step gradient. EM imaging of exosome samples before and after sucrose step gradient purification showed that this procedure yields a pure exosome preparation free of non-exosomal vesicles, subcellular organelles, or debris (Fig. 2A). These observations further indicate that the exosomes isolated using this protocol are secreted into the brain extracellular space rather than contaminating intracellular vesicles released during the experimental procedure. Exosomal content depends on the cell type or tissue it is secreted from (3); however, all exosomes contain specific markers such as TSG101, an element of the ESCRT protein complex controlling exosome formation (19), and the endosomal flotillin (3, 20). Measurements of the levels of these exosomal markers and AChE activity levels in exosomes are commonly used tools to quantify exosomes (15, 21). Therefore, the presence of exosomes and the amount of exosomes in each sucrose gradient fraction (fractions a–g) were determined by measuring the pro-

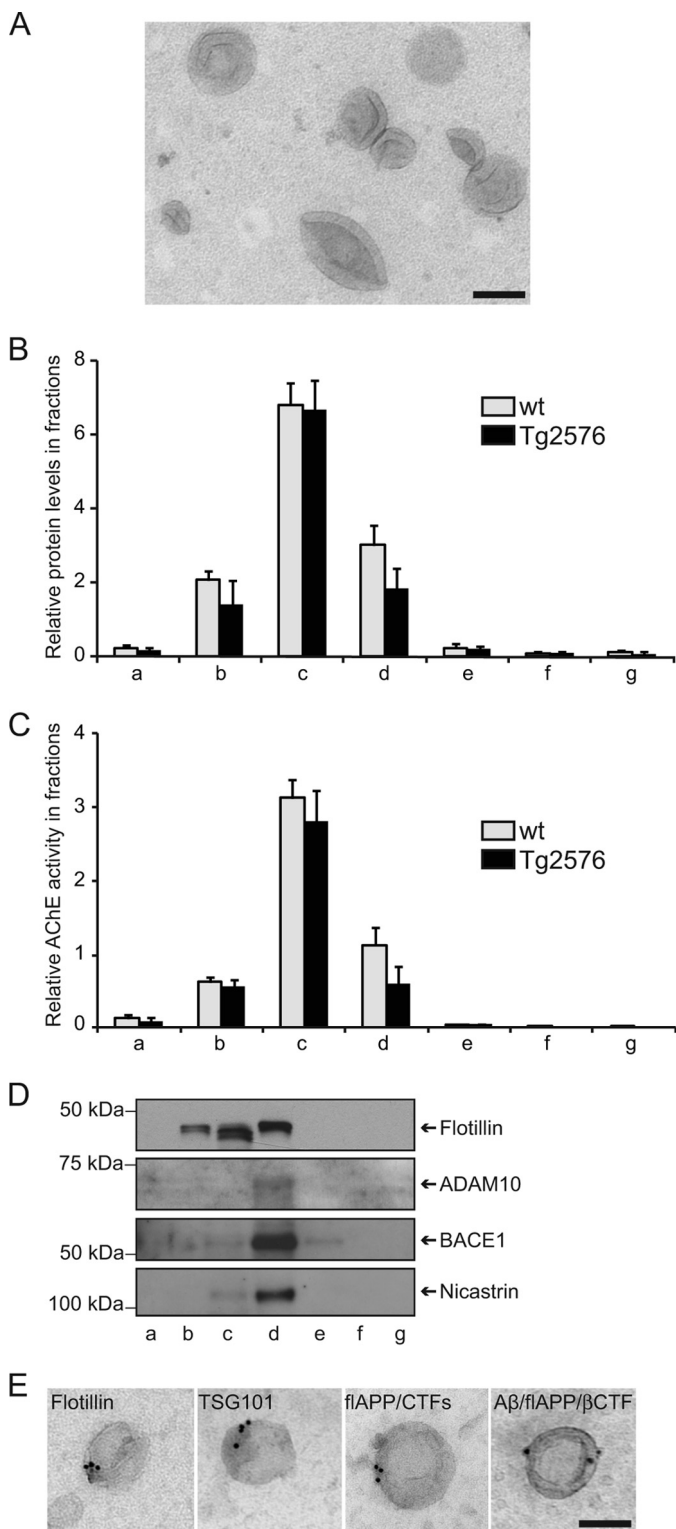


**FIGURE 1. Brain exosome isolation experimental flow chart.** The steps of the experimental procedure (right) designed to isolate and purify brain exosomes are described along with the associated objectives of each step of the procedure (left).

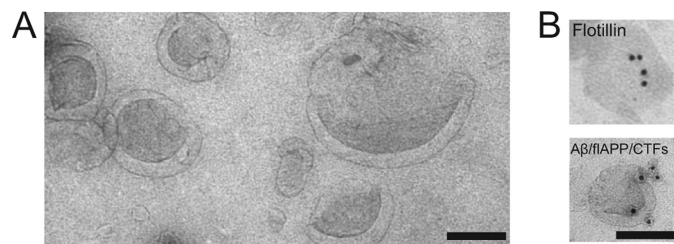
tein content and AChE activity level, by Western blot analysis using antibodies to exosomal markers and by EM.

EM analysis of the content of the sucrose step gradient fractions identified typical exosome-like cup-shaped vesicles ranging in size from 50 to 150 nm in three of the fractions (b–d), but not in the other fractions (a and e–g) (data not shown). The brain exosomes were collected from sucrose layers where material with density higher than 1.07 (0.60 M glucose fraction layer) and lower than 1.17 (1.30 M glucose fraction layer) segregated during the fractionation procedure. The three fractions contained proteins that accounted for 94.92% of the total exosomal protein content (Fig. 2B) and for 97.97% of the total exosomal AChE activity (Fig. 2C) and were immunoreactive with anti-flotillin antibody (Fig. 2D). Immuno-EM studies of isolated exosomes, prior to their purification on a sucrose step gradient column, showed that the isolated exosomes contained the exosomal protein markers flotillin and TSG101 (Fig. 2E). Using this procedure, we found that isolation of exosomes from mouse brain tissue frozen and kept at  $-80^{\circ}\text{C}$  for 8 months yielded the same number of exosomes with the same morphology as isolation of exosomes from freshly removed brain.





**FIGURE 2. Characterization of exosomes isolated from murine hemibrains.** *A*, wide-field EM imaging showed multiple mouse brain exosomes of sizes ranging from 50 to 150 nm and no other structures or cellular debris. Scale bar = 100 nm. *B* and *C*, exosomes isolated from the brains of WT and APP transgenic (Tg2576) mice were found in sucrose step gradient fractions b–d. Exosomal protein content (*B*) and exosomal AChE activity levels (*C*) standardized to total brain protein content were not different between transgenic and non-transgenic brain exosomes collected from fractions b–d. *D*, Western blotting demonstrated the presence of the exosomal marker flotillin in fractions b–d. The enzymes  $\alpha$ -secretase (ADAM10),  $\beta$ -secretase (BACE1), and  $\gamma$ -secretase (nicastrin) were also detected in exosomal fractions. *E*, immuno-EM showed that exosomes isolated from mouse brains were identified by antibodies to the exosomal markers flotillin and TSG101 and were immunoreactive to antibodies that react with flAPP and APP  $\beta$ -CTF (C1/6.1) and with flAPP, APP  $\beta$ -CTF, and A $\beta$  (4G8). Scale bar = 100 nm.



**FIGURE 3. Exosomes isolated from frozen human brain are immunoreactive to anti-flotillin and anti-flAPP, APP CTFs and A $\beta$ .** *A*, wide-field EM imaging showed multiple human brain exosomes of sizes ranging from 50 to 150 nm and no other structures or cellular debris. *B*, immuno-EM showed that exosomes isolated from human brains were identified by antibodies to the exosomal marker flotillin and were immunoreactive to antibodies that react with flAPP and APP metabolites (6E10). Scale bars = 100 nm.

*Frozen Human Brain Tissues Yield Intact Secreted Exosomes*—Using the same procedure designed for isolating exosomes from mouse brain tissue, we isolated exosomes from Brodmann area 9 of brain tissue from an AD patient and a neuropathologically normal control (frozen and kept at  $-80^{\circ}\text{C}$  for long periods of time). EM analysis showed exosomes with the same morphology as exosomes isolated from freshly removed mouse brain (Fig. 3).

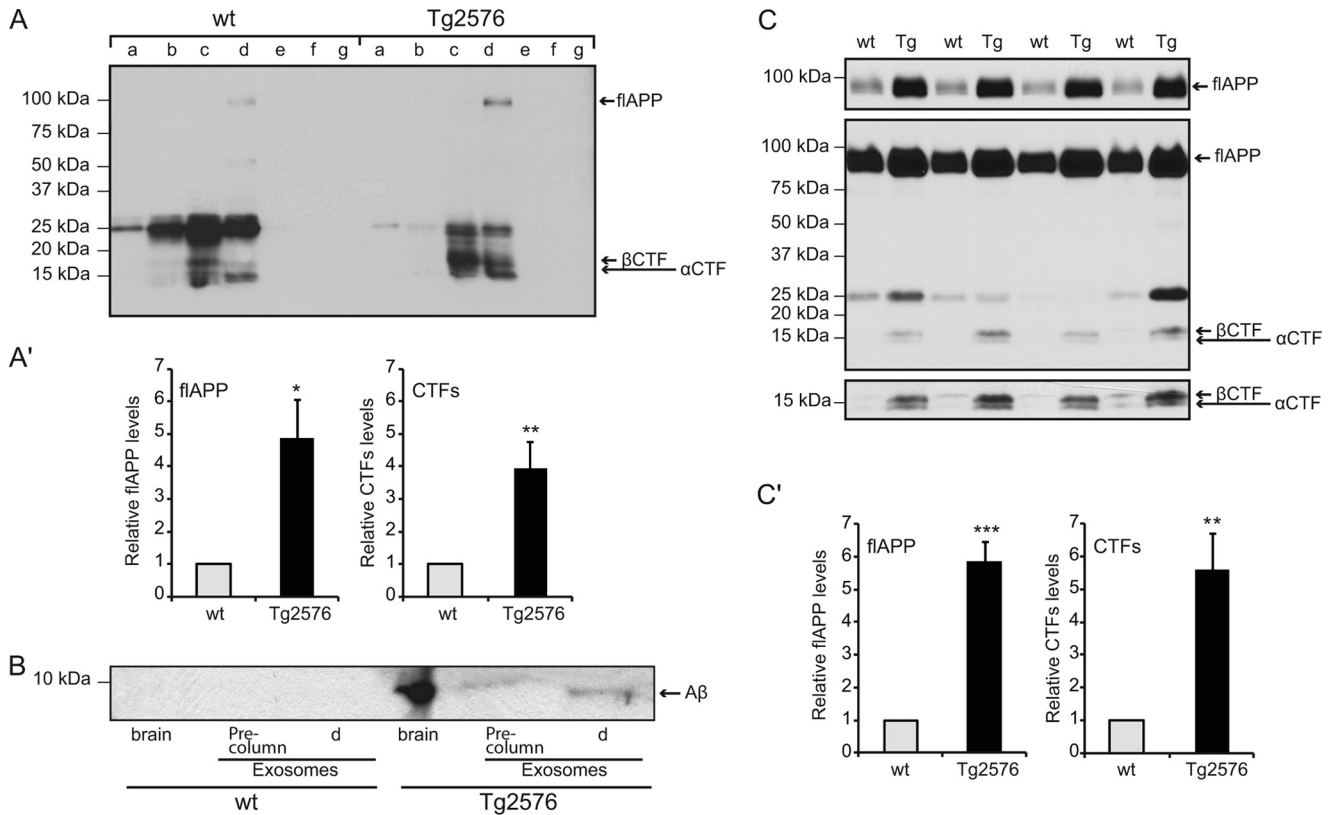
*Secreted Brain Exosomes Contain Enzymes That Cleave Both flAPP and APP CTFs*—Western blot analysis of brain exosomes (Fig. 2*D*) showed that an  $\alpha$ -secretase (ADAM10), a  $\beta$ -secretase (BACE1), and nicastrin (a component of the  $\gamma$ -secretase complex) were present in fractions also containing flotillin and AChE activity, but not in fractions lacking either flotillin (Fig. 2*D*) or AChE activity (Fig. 2*C*). These data confirm *in vitro* studies showing that exosomes secreted by cultured cells contain the three APP-cleaving enzymes (11, 22).

*Secreted Brain Exosomes Contain flAPP and APP CTFs*—Immuno-EM imaging analysis of Tg2576 and wild-type control brain exosomes showed immunoreactivity with antibodies that identify flAPP, APP CTFs, and A $\beta$  in exosomes that were immunoreactive with antibodies to the exosomal markers flotillin and TSG101 (Fig. 2*E*). In addition, Western blot analysis of brain exosomes (Fig. 4*A*) showed that flAPP and APP CTFs were present in fractions b–d, which also contained flotillin and AChE activity, but not in fractions a and e–g, which lacked either flotillin (Fig. 2*D*) or AChE activity (Fig. 2*C*). Western blot analysis confirmed the immuno-EM observation (Fig. 2*E*) that A $\beta$  was associated with exosomes (Fig. 4*B*). These results show that brain exosomes transport flAPP, APP CTFs, and A $\beta$  to the extracellular space *in vivo*.

To test the effect of APP overexpression in Tg2576 mice on the number of exosomes secreted, we quantified exosomal protein content and AChE activity levels standardized to total brain protein content in all of the sucrose gradient fractions and did not find a difference between Tg2576 and wild-type control mice (Fig. 2, *B* and *C*). These data reveal that Tg2576 and wild-type control mice secrete the same number of exosomes into the brain extracellular space. Western blot analysis of flAPP and APP CTFs in brain homogenates and in brain exosomes of

identified by antibodies to the exosomal markers flotillin and TSG101 and were immunoreactive to antibodies that react with flAPP and APP  $\beta$ -CTF (C1/6.1) and with flAPP, APP  $\beta$ -CTF, and A $\beta$  (4G8). Scale bar = 100 nm.

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**FIGURE 4. Exosomes isolated from brain tissues of Tg2576 and wild-type control mice contain flAPP, APP CTFs, and A $\beta$ .** flAPP and APP CTFs were revealed by Western blot analysis of exosome lysates (A) and brain homogenates (C) of Tg2576 (Tg) and WT mice with antibody C1/6.1. Protein bands were quantified and are presented as the ratio between Tg2576 and wild-type levels of either flAPP or APP CTFs. flAPP in the brains (C') and brain exosomes (A') of Tg2576 mice was 5.8 times ( $p \leq 0.0001$ , Student's *t* test;  $n = 3$ ) and 4.9 times ( $p = 0.0177$ , Student's *t* test;  $n = 3$ ) higher, respectively, than in wild-type control mice. APP CTFs levels in the brains (C') and brain exosomes (A') of Tg2576 animals were 5.6 times ( $p = 0.0036$ , Student's *t* test;  $n = 3$ ) and 4.0 times ( $p = 0.0056$ , Student's *t* test;  $n = 3$ ) higher, respectively, than in wild-type control mice. Western blot analysis with antibody 6E10 showed A $\beta$  associated with exosomes in the brains of Tg2576 mice (B). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

Tg2576 and littermate non-transgenic mice was conducted to characterize the effect of APP overexpression in the Tg2576 brain on exosomal flAPP and APP CTFs levels. Quantitation of protein bands revealed that the brains of Tg2576 mice contain 5.8 times more flAPP and 5.6 times more APP CTFs than the brains of littermate non-transgenic mice (Fig. 4, C and C') and that Tg2576 brain exosomes contain 4.9 times more flAPP and 4.0 times more APP CTFs than littermate non-transgenic brain exosomes (Fig. 4, A and A'). These data show that higher exosomal flAPP and APP CTFs levels rather than greater number of secreted exosomes with normal flAPP and APP CTFs levels are responsible for the higher levels of flAPP and APP CTFs secreted into the extracellular space via exosomes in the brains of Tg2576 mice.

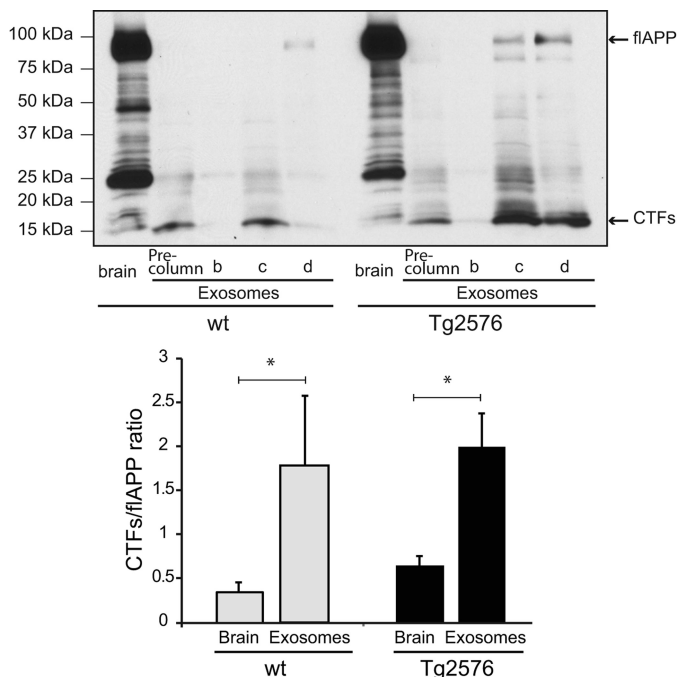
**Brain Exosomes Are Enriched with APP CTFs Compared with Brain Tissue**—A difference in the ratio of APP CTFs to flAPP in exosomes compared with brain homogenates was demonstrated by Western blot analysis with antibody C1/6.1 (17) (Fig. 5). Exosomes retrieved prior to purification on a sucrose step gradient column showed low levels of flAPP and APP CTFs compared with fractions b–d collected from the sucrose gradient column because smaller volumes were loaded on the gel compared with the volumes loaded from the fractions collected from the sucrose gradient column. Comparison of the ratio of APP CTFs to flAPP in brain homogenates and in brain exo-

somes showed that the ratio was higher in brain exosomes compared with brain homogenates in both Tg2576 and wild-type non-transgenic mice. The APP CTF:flAPP ratios were similar in samples obtained from Tg2576 and wild-type control mice (Fig. 5), indicating that the cellular levels of flAPP and APP CTFs do not regulate the ratio between the full-length protein and its metabolites in exosomes. These results show that exosomes secreted into the extracellular space of the brains of mice, both Tg2576 and wild-type, are enriched with APP CTFs.

## DISCUSSION

We developed and validated a novel protocol designed to isolate exosomes from the extracellular space of human and murine brain tissue. We found that this procedure yields vesicles that match in shape, size, density, and protein content exosomes previously isolated from bodily fluids or from the conditioned media of various types of cell cultures (3, 15). As was previously shown for human urine (23), cerebrospinal fluid (24), and plasma (25), intact exosomes can be isolated from frozen tissues. EM analysis revealed no difference between exosomes isolated from human brain tissues frozen for several years at  $-80^\circ\text{C}$ , mouse brains frozen for long periods of time at  $-80^\circ\text{C}$ , and freshly isolated mouse brains.

We have shown that murine brain exosomes contain flAPP and the APP metabolites APP CTFs and A $\beta$ , as was shown



**FIGURE 5. Brain exosomes are enriched with APP CTFs.** Shown are the results from Western blot analysis with antibody C1/6.1 of brain homogenates and exosomes isolated from the brains of WT and Tg2576 mice retrieved prior to purification on a sucrose step gradient column (*Pre-column*) or from fractions b–d collected from the sucrose gradient column. Protein bands were quantified and are presented as the ratio between APP CTFs and flAPP in brain homogenates and exosomes. The APP CTF:flAPP ratio was higher in exosomes compared with brain homogenates in the brains of wild-type control mice ( $p = 0.025$ ) and Tg2576 mice ( $p = 0.027$ ) (analysis of variance with Fisher's least significant difference post-hoc test;  $n = 3$ ). The APP CTF:flAPP ratio in Tg2576 brain exosomes compared with wild-type control brain exosomes was not different ( $p > 0.05$ ). \*,  $p < 0.05$ .

previously for exosomes isolated from the conditioned media of neuronal cells cultured *in vitro* (11). We isolated secreted brain exosomes from the hemi-brains of Tg2576 and wild-type control mice to study the effects of APP overexpression on brain exosome content and the number of exosomes secreted in the brain. Our results show that higher APP expression in the brains of Tg2576 mice enhances flAPP and APP CTF secretion into the extracellular space through the exosome secretory pathway by increasing the content of these proteins in individual brain exosomes, but not by increasing the number of secreted brain exosomes. In addition, we showed that exosomes isolated from the brains of mice, both Tg2576 and non-transgenic control mice, are enriched with APP CTFs compared with brain tissue. Considering that brain homogenates contain both cellular and extracellular materials, including exosomes, the contribution of the cellular APP CTF:flAPP ratio is predicted to be lower, suggesting an even higher level of APP CTF enrichment in exosomes. These novel results imply the existence of a set of mechanisms driving APP CTF enrichment in exosomes.

Exosome protein composition includes both a ubiquitous composition and a cell type-specific composition. The exosomal proteins that have been identified are found in the parental cell (6). The initial step in exosome biogenesis is intraluminal vesicle formation by inward budding of the MVB membrane inside the cell. The resulting vesicles contain cytosol and the

exposed internal membrane of the MVB at their surface. Thus, the membrane of the exosome contains proteins present in the endosomal membrane, and its lumen contains cytosolic proteins, mirroring the variety and amounts present in the releasing cell. The transmembrane glycoprotein APP is transported from the Golgi to the cell membrane by the constitutive secretory pathway. flAPP and APP CTFs undergo endocytosis via clathrin-coated vesicles and are trafficked to various endosomal compartments, including MVBs (26). APP internalized via endocytosis is either recycled back to the cell membrane or directed to the endosomal-lysosomal pathway, where APP catabolism occurs (27).  $\beta$ -Cleavage of APP occurs in early endosomes, followed by routing of flAPP, APP CTFs, and  $A\beta$  to MVBs. APP CTFs and  $A\beta$  accumulate in MVBs of neurons in normal mouse, rat, and human brain, and this accumulation increases with age in APP transgenic mice and human AD brains (28, 29). Subsequently, flAPP, APP CTFs, and  $A\beta$ , but not APP N-terminal fragments, are secreted from the cells in association with exosomes (11, 30).

Although the endosomal pathway and MVBs are essential organelles for APP metabolism and APP metabolites can be secreted via exosomes, processing of APP and/or APP CTFs occurs also in exosomes. In addition to flAPP and APP CTFs, key members of the secretase family of proteases (ADAM10, BACE1, presenilin-1, presenilin-2, and nicastrin) are localized in exosomes (Fig. 2D) (22, 31). It was demonstrated that in exosomes secreted into the medium of SKNSH-SY5Y cells overexpressing wild-type APP, flAPP staining only slightly increases over time, but APP CTF staining strongly increases with time (11). It was shown that inhibition of  $\gamma$ -secretase results in a significant increase in the amount of  $\alpha$ - and  $\beta$ -secretase cleavage, increasing the amount of APP CTFs contained within exosomes (22). Immuno-EM using antibodies directed to cytoplasmic or extracellular epitopes of APP revealed that the exosomes are oriented with the cytoplasmic side facing inward (32). Therefore, cleavage of APP results in capture of APP CTFs inside exosomes. Thus, the enrichment of APP CTFs in exosomes isolated from the brains of Tg2576 and wild-type control mice results from endosomal APP CTFs that were captured and carried by exosomes combined with metabolism of flAPP in the exosomes. Contrary to flAPP and APP CTFs, only a minute fraction of total  $A\beta$  (<1%) is associated with exosomes (30). Exosomes are generated by inward budding of the MVB membrane and therefore contain cytosolic material, but not proteins present in the lumen of MVBs such as  $A\beta$ . Furthermore, the orientation of APP in the membrane of exosomes would result in the secretion of exosome-generated  $A\beta$  into the extracellular space, but not into the vesicles. Moreover, the insulin-degrading enzyme, the major  $A\beta$ -degrading enzyme, is found in exosomes, suggesting an additional pathway for the regulation of extracellular  $A\beta$  (33, 34).

Exosomal proteins were found to accumulate in amyloid plaques in the brains of AD patients (30), and it was therefore proposed that exosomes participate in the pathogenesis of AD. Extensive research suggests that several APP metabolites are toxic to neuronal cells. A central pathological feature of AD is the accumulation of  $A\beta$  in the brain, and it was suggested that  $A\beta$  has an important role in the pathogenesis of neuronal dys-



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function in the disease (reviewed in Refs. 35–37). Although A $\beta$  is prone to misfolding and builds up fibrillar aggregates that are neurotoxic (38, 39), both *in vitro* and *in vivo* reports describe a potent neurotoxic activity for soluble, non-fibrillar, oligomeric assemblies of A $\beta$  (reviewed in Refs. 40 and 41). Recent *in vitro* and *in vivo* studies suggest that other APP metabolites,  $\beta$ -secretase-derived APP CTFs and possibly the N-terminal fragment of APP (secreted APP $\beta$ ), are neurotoxic and cause memory loss (42–45). The presence of precursors to amyloidogenic proteins and amyloidogenic proteins within exosomes suggests that exosomes can release amyloidogenic material and transmit it between cells, playing a pathogenic role in neurodegenerative diseases. Similar to A $\beta$ , the normal prion protein (PrP<sup>C</sup>) and the abnormally folded infectious scrapie (PrP<sup>Sc</sup>) are associated with exosomes. Prion diseases are infectious neurodegenerative disorders linked to the accumulation in the central nervous system of PrP<sup>Sc</sup>. Exosome-associated PrP<sup>Sc</sup> released by PrP-expressing cells elicits conversion of endogenous PrP<sup>C</sup> to PrP<sup>Sc</sup> when incubated with uninfected recipient cells (46). Enrichment of PrP<sup>C</sup> was also found in exosomes derived from ovine cerebral spinal fluid (47).  $\alpha$ -Synuclein aggregation plays a central role in Parkinson disease pathology, and it was demonstrated that exosomes released from  $\alpha$ -synuclein-overexpressing SH-SY5Y cells contain  $\alpha$ -synuclein. These exosomes are capable of efficiently transferring  $\alpha$ -synuclein to normal SH-SY5Y cells (48, 49). Exosome-associated tau phosphorylated at Thr-181 (AT270), an established phosphorylated tau biomarker for AD, was found in tissue culture media and in human cerebrospinal fluid samples, suggesting that exosome-mediated secretion of phosphorylated tau may play a role in the abnormal processing of tau and in the genesis of elevated cerebrospinal fluid tau in early AD (50). Furthermore, exosomes isolated from the plasma of a murine transfer model of the systemic AA (amyloid A) amyloidosis contain AA amyloid oligomers derived from serum amyloid A and amyloid-enhancing factor activity and could transmit systemic AA amyloidosis (51). These data suggest that exosomes may contribute to intercellular membrane exchange and the spread of aggregation-prone proteins throughout the organism.

Although these findings suggest a pathogenic role for exosomes, exosomes may have a protective function by relieving the cells from toxic accumulation of peptides such as prion proteins,  $\alpha$ -synuclein, as well as APP CTFs that accumulate intracellularly and disrupt the normal function of the endosomal-lysosomal system to degrade proteins. Thus, we hypothesize that exosome secretion plays a pleiotropic role in the brain, both beneficial to the cell by discarding APP CTFs and also harmful to the brain by contributing to the extracellular buildup of toxic APP metabolites. The secretion of exosomes loaded with APP CTFs into the extracellular space can increase A $\beta$  secretion into the extracellular space and speed up A $\beta$  deposition in amyloid plaques, a landmark of AD pathology.

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