

## RESEARCH ARTICLE

# Extracellular vesicles: where the amyloid precursor protein carboxyl-terminal fragments accumulate and amyloid- $\beta$ oligomerizes

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## Abstract

Pleiotropic roles are proposed for brain extracellular vesicles (EVs) in the development of Alzheimer's disease (AD). Our previous studies have suggested a beneficial role for EVs in AD, where the endosomal system in vulnerable neurons is compromised, contributing to the removal of accumulated material from neurons. However, the involvement of EVs in propagating AD amyloidosis throughout the brain has been considered because the amyloid- $\beta$  precursor protein (APP), APP metabolites, and key APP cleaving enzymes were identified in association with EVs. Here, we undertook to determine whether the secretase machinery is actively processing APP in EVs isolated from the brains of wild-type and APP overexpressing Tg2576 mice. We found that full-length APP is cleaved in EVs incubated in the absence of cells. The resulting metabolites, both  $\alpha$ - and  $\beta$ -APP carboxyl-terminal fragments and APP intracellular domain accumulate in EVs over time and amyloid- $\beta$  dimerizes. Thus, EVs contribute to the removal from neurons and transport of APP-derived neurotoxic peptides. While this is potentially a venue for propagation of the pathology throughout the brain, it may contribute to efficient removal of neurotoxic peptides from the brain.

**Abbreviations:** A $\beta$ , amyloid- $\beta$ ; AD, Alzheimer's disease; AICD, APP intracellular domain; APP, Amyloid- $\beta$  precursor protein; APP-CTFs, APP carboxyl-terminal fragments; DMEM, Dulbecco's Modified Eagle Medium; EVs, Extracellular vesicles; flAPP, full-length amyloid- $\beta$  precursor protein; PS1, Presenilin-1; PS2, Presenilin-2.

Rocío Pérez-González and Yohan Kim contributed equally to this study.

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**KEYWORDS**A $\beta$  oligomers, Alzheimer's disease, APP-CTFs, APP metabolism, exosomes

## 1 | INTRODUCTION

Extracellular vesicles (EVs) are highly stable membranous structures that are secreted by most cell types and can be subsequently taken up by recipient cells (reviewed in 1). The various species of EVs have different origins. While microvesicles bud directly from the plasma membrane, exosomes have an endosomal origin because they are formed by invagination of the membrane of late endosomes (also called multivesicular bodies).<sup>2</sup> Given that sorting EVs derived from a particular biogenesis pathway remains difficult, the general term EVs will be used here to refer to small EVs enriched with exosomes, following the International Society for EVs recommendations.<sup>3</sup> EV secretion was originally described as a complementary process to the lysosomal and proteosomal degradative pathways to discard obsolete membrane and cytosolic proteins in differentiating reticulocytes.<sup>4</sup> Accordingly, our previous research has supported a beneficial role for EVs in vulnerable neurons with endosomal pathology, one of the earliest insults observed in Alzheimer's disease (AD),<sup>5</sup> by relieving the cells from toxic waste that was not efficiently degraded intracellularly.<sup>6,7</sup> However, the identification of full-length amyloid- $\beta$  precursor protein (flAPP) and amyloid- $\beta$  precursor protein (APP) metabolites in EVs<sup>8-11</sup> has implicated EVs in AD amyloid pathology by carrying and spreading pathology throughout the brain. The accumulation of the amyloid- $\beta$  (A $\beta$ ) peptide in extracellular senile plaques is one of the pathological hallmarks of AD.<sup>12</sup> A $\beta$  is generated through sequential processing of flAPP, which undergoes proteolytic cleavage by  $\alpha$ - and  $\beta$ -secretases to produce  $\alpha$ - and  $\beta$ -carboxyl-terminal fragments (APP-CTFs), respectively.  $\beta$ -APP-CTF (also referred to as C99) is the source of A $\beta$  following cleavage by  $\gamma$ -secretase,<sup>13</sup> which also results in the generation of the APP intracellular domain (AICD).<sup>14</sup> Processing of flAPP occurs in the plasma membrane and in endosomes<sup>15</sup> and APP metabolites can be incorporated into EVs<sup>16</sup> and released from the cells into the extracellular environment. However, it was suggested that only a minute fraction of total A $\beta$  is associated with EVs,<sup>9,16</sup> unlike APP-CTFs, which are abundantly present within EVs.<sup>8-11,17</sup> Accumulation of  $\beta$ -APP-CTFs in specific brain regions is a common early process in different AD mouse models that may contribute to AD pathology (reviewed in 18). Multiple studies have shown that  $\beta$ -APP-CTFs are neurotoxic, causing neuronal endosomal-lysosomal abnormalities<sup>19-21</sup> leading to neuronal cell death and memory loss.<sup>22-28</sup> Therefore, the transport of  $\beta$ -APP-CTF via EVs throughout the brain may result in the uptake of this peptide by neurons with

deleterious effects.  $\beta$ -APP-CTFs are also a source of A $\beta$  peptides that will assemble into oligomeric forms prior to the formation of senile plaques.<sup>29</sup> These oligomeric forms of A $\beta$  are considered a major culprit in the synaptic dysfunction and neuronal loss that characterize AD.<sup>30-32</sup> The process of oligomerization and subsequent aggregation of A $\beta$  into fibrils can be facilitated by EVs, as it has been shown that they can act as amyloid “seeding” sites.<sup>33,34</sup> Further, the identification in EVs of key enzymes involved in APP metabolism<sup>9,10,35,36</sup> has suggested that EVs are a potential site for the de novo generation of APP-CTFs and A $\beta$ . Because EVs are oriented with the cytoplasmic-side facing inward,<sup>37</sup> the same orientation as in the plasma membrane, it was hypothesized that cleavage of flAPP would result in capture of APP-CTFs in the membrane of EVs and in the secretion of EV-generated A $\beta$  into the extracellular space. Here, we investigated whether flAPP is enzymatically cleaved in EVs in the absence of cells.

## 2 | MATERIALS AND METHODS

### 2.1 | Mice

Brains were derived from wild-type C57/B6 mice at the age of 12 months and from transgenic mice overexpressing human APP with the K670N/M671L Swedish double mutation (Tg2576)<sup>38</sup> at 4 months of age. Both females and males were used for all analyses. 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.

### 2.2 | Brain EV isolation

Brain EVs were isolated and purified as we have previously described.<sup>9</sup> Briefly, frozen brain tissues were treated with 20 units/mL of papain (Worthington, Lakewood, NJ, USA) in Hibernate A solution (HA, 3.5 mL/sample; BrainBits, Springfield, IL, USA) for 15 minutes at 37°C. The brain tissue was gently dissociated in 6.5 mL of cold HA supplemented with protease inhibitors, centrifuged at 300 *g* for 10 minutes at 4°C to discard the cells, and the supernatant was sequentially filtered through a 40  $\mu$ m mesh filter (BD Biosciences, San Jose, CA, USA) and a 0.2  $\mu$ m syringe filter (Corning Life Sciences, Teterboro, NJ, USA). The filtrates were sequentially centrifuged at 4°C, at 2000 *g* for 10 minutes and

10 000 *g* for 30 minutes to discard membranes and debris, and at 100 000 *g* for 70 minutes to pellet the EVs. The EV pellet was resuspended in 60 mL of cold PBS (Thermo Fisher Scientific), and centrifuged at 100 000 *g* for 70 minutes at 4°C. The washed EV pellet was resuspended in 2 mL of 0.95 M sucrose solution and inserted inside a sucrose step gradient column (six 2-mL steps from 2.0 to 0.25 M sucrose). The sucrose step gradient was centrifuged at 200 000 *g* for 16 hours and fractions were collected from the top of the gradient. The fractions were diluted in cold PBS and centrifuged at 100 000 *g* for 70 minutes for pellet collection.

## 2.3 | Incubation of isolated EVs at 37°C

Brain EV pellets from fractions C and D of the sucrose step gradient<sup>9</sup> were resuspended in 30  $\mu$ L each of Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific) and combined. The pooled EVs were divided into experimental groups incubated for the indicated times (0, 1, 4, 24, 48, or 72 hours), in the absence or presence of either the  $\gamma$ -secretase inhibitor L685,458, or A $\beta$ -degrading enzyme inhibitors. The same volume of DMEM containing 2X EDTA (except in the experiments using inhibitors of A $\beta$ -degrading enzymes), without or supplemented with  $\gamma$ -secretase inhibitor or an A $\beta$ -degrading enzyme inhibitor, was added to the EV suspensions. The  $\gamma$ -secretase inhibitor, L685,458 (Tocris Bioscience, Minneapolis, MN, USA) was used at the final concentration of 10  $\mu$ M. Inhibitors of A $\beta$ -degrading enzymes (thiorphan [Cayman] and phosphoramidon [Sigma-Aldrich]) were added to the EV suspensions at the final concentrations of 10 and 100  $\mu$ M, respectively. Though each can inhibit multiple metalloproteases, at these concentrations thiorphan is selective for neprilysin over endothelin-converting enzymes (ECEs) and phosphoramidon inhibits neprilysin and ECEs. At time 0 hour EVs were immediately lysed in 2X RIPA buffer (1% Triton-X, 1% Sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl pH 7.4, and 1 mM EDTA) supplemented with 2X Halt Protease inhibitors (Thermo Fisher Scientific) and 1X EDTA (Thermo Fisher Scientific). At times 1, 4, 24, 48, and 72 hours EVs were placed in a 37°C bath incubator for the time periods indicated and subsequently lysed in 2X RIPA buffer. All lysates were sonicated for 45 seconds, placed on ice for 20 minutes with vortex-mixing every 5 minutes and kept at -80°C until analysis.

## 2.4 | Preparation of A $\beta$ peptide solution

For preparation of the 10  $\mu$ M peptide stock, lyophilized A $\beta$ 40 peptide (2  $\mu$ g) was dissolved and equilibrated in dimethyl sulfoxide (Sigma-Aldrich) for 15 minutes at room temperature with vortex-mixing every 3 minutes. Subsequently,

the 10  $\mu$ M of A $\beta$ 40 stock was diluted in DMEM to make a 300 nM of A $\beta$ 40 solution, which was further diluted to the final concentration of 10 nM in the solution used for the Western blot analysis.

## 2.5 | Western blot analysis

The same amount of EV proteins was separated by 4%-20% Tris-HCl gel electrophoresis (Criterion precast gel, Bio-Rad, Hercules, CA, USA) and transferred onto PVDF membranes (Immobilon, Millipore). Membranes were incubated with antibodies to HSC70 (1:1000, Cat# sc-7298, RRID:AB\_62776; Santa Cruz Biotechnology), CD63 (1:1000, Cat# ab217345, RRID:AB\_2754982; Abcam), APP and APP-CTFs (C1/6.1,<sup>39</sup> 1:1000), BACE1 (1:1000, Cat# 200-401-984, RRID:AB\_2243187; Rockland), ADAM10 (1:1000, Cat# AB19026, RRID:AB\_2242320; Millipore), Nicastrin (1:1000, Cat# MAB5556, RRID:AB\_2235791; Millipore). The antibodies to the subunits of the  $\gamma$ -secretase complex: PS1 (N-terminal, 1:1000), PS2 (N-terminal, 1:50), APH1a (C-terminal, 1:1000), and APH1b (C-terminal, 1:1000) were a kind gift from Dr Paul Fraser, University of Toronto. The PEN-2 antibody (N-terminal, 1:2500) was a kind gift from Dr Thinakaran, University of Chicago. The secondary antibodies used were HRP-conjugated anti-rabbit or anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA, USA). The membranes were incubated in chemiluminescent fluid (Pierce, Rockford, IL, USA) and chemiluminescence was visualized on X-ray films. For identification of A $\beta$  dimers, the same amount of EV proteins was separated by 16.5% tris-tricine gels, blotted with a 1:1 mixture of the 6E10 (1:1000; Cat# 803001, RRID:AB\_2564653; BioLegend) and 4G8 (1:1000; Cat# SIG-39220, RRID:AB\_662812; Covance) antibodies on PVDF. 6E10 and 4G8 antibodies are generated against different epitopes within A $\beta$ .<sup>40</sup> For visualization of A $\beta$  monomers and AICD, EV proteins separated by 16.5% tris-tricine gels were transferred onto Nitrocellulose (Bio-Rad), which was subsequently boiled in PBS for 4 minutes, followed by blotting in 6E10 for A $\beta$  monomer or C1/6.1 for AICD detection. Protein bands were quantified using ImageJ (National Institute of Health (NIH), Bethesda, MD, USA).

## 2.6 | ELISA

A $\beta$  peptides were analyzed by sandwich ELISA with specific mouse monoclonal antibodies, as described previously.<sup>41</sup> For human A $\beta$ 40 measurement, antibody MM27-33.1.1 (raised against human A $\beta$  1-16) was used for capture and MM32-13.1.1-HRP (raised against A $\beta$ 35-40) for detection. For A $\beta$ 42, anti-A $\beta$ 42 antibody MM26-2.1.3 (A $\beta$ 35-42) was used for capture and HRP-conjugated 4G8 antibody

(A $\beta$ 17-24) was used for detection. A $\beta$  antibodies except for 4G8 (Covance, Princeton, NJ, USA) were from Mayo Clinic (Rochester, MN, USA).

## 2.7 | Data analysis and graphing

The statistical and graphing software Prism 5.01 (GraphPad Software, La Jolla, CA, USA) was used for data analysis and graphical representation. For column graphs, column bars represent means  $\pm$  SEM. To compare the relative changes in the levels of  $\alpha$ - and  $\beta$ -APP-CTFs, A $\beta$  monomers, A $\beta$  dimers and AICD, analysis of variance (one-way ANOVA) followed by Tukey's post hoc multiple comparison was performed (Figures 2 and 3).

## 3 | RESULTS

### 3.1 | APP present in EVs is enzymatically processed to generate APP-CTFs

In order to study whether flAPP is cleaved in situ in EVs, we investigated the presence in EVs of the  $\alpha$ -secretase ADAM10,<sup>42</sup> the  $\beta$ -secretase BACE1,<sup>43</sup> and all the components of the  $\gamma$ -secretase, a membrane protein complex comprised of Presenilin-1 (PS1) or Presenilin-2 (PS2), APH-1a or APH-1b, Nicastrin, and PEN-2.<sup>44</sup> In addition to the confirmation of the presence of previously identified secretases, we found all the  $\gamma$ -secretase complex subunits in EVs isolated from wild-type mouse brain (Figure 1A).

We then tested whether these secretases are functional in situ, generating APP-CTFs. EVs isolated from the brain of wild-type mice were incubated in DMEM in the absence of cells at 37°C for different periods of time. Following incubation, the EVs with the medium in which they were incubated were lysed in RIPA buffer and analyzed by Western blotting. The results showed that flAPP is enzymatically processed to generate  $\alpha$ - and  $\beta$ -APP-CTFs in a time-dependent manner (Figure 1B), confirming the de novo generation of APP-CTFs in brain EVs.

### 3.2 | APP-CTFs are further processed into AICD in EVs and A $\beta$ peptides oligomerize

We next determined whether the  $\gamma$ -secretase complex in EVs is enzymatically active to generate A $\beta$  and AICD. Given the low A $\beta$  production in the brain of wild-type mice, we isolated EVs from the brain of APP overexpressing transgenic mice, Tg2576,<sup>38</sup> at an age prior to the initiation of amyloid deposition. We first corroborated that APP processing occurs in EVs isolated from the brain of Tg2576 mice during

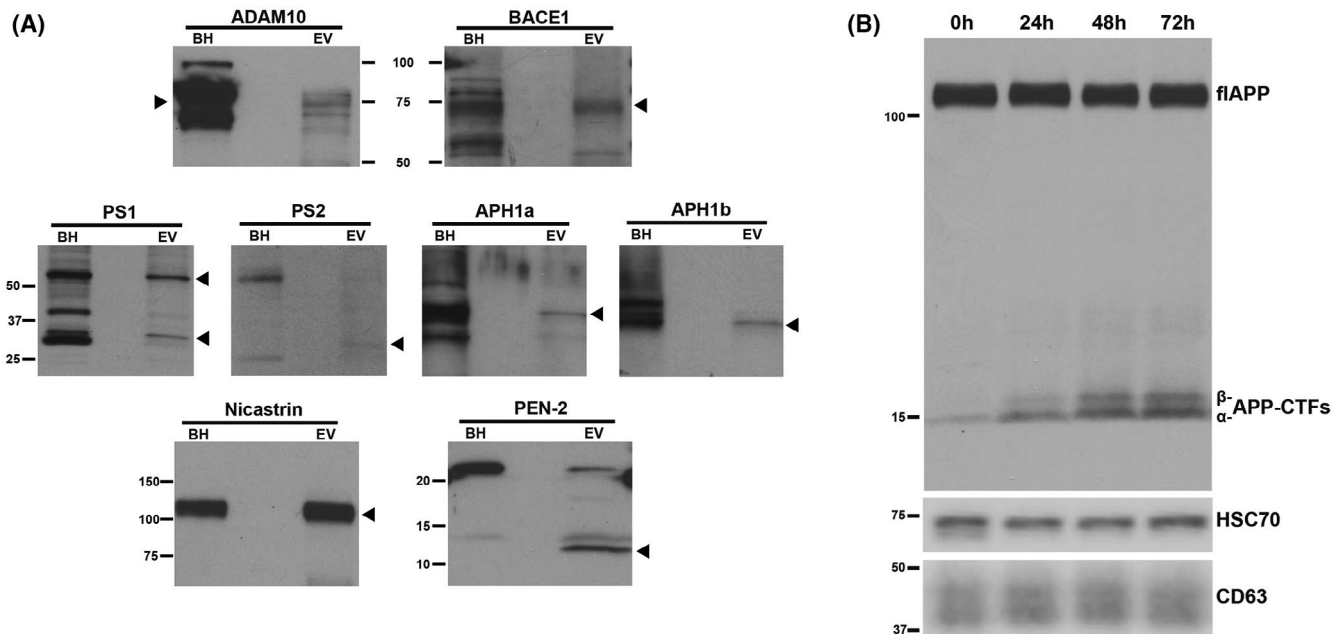
a 24-hour incubation, generating both  $\alpha$ - and  $\beta$ -APP-CTFs (Figure 2A,B) at levels higher than in EVs isolated from the brain of wild-type mice (Figure 2A). Incubation in the presence of the  $\gamma$ -secretase inhibitor, L685,458 had no significant effect on the levels of the APP-CTFs (Figure 2A). An indication for the  $\gamma$ -secretase cleavage of the APP-CTFs in the incubated EVs is the increase in the level of AICD following 24-hour incubation and the absence of the increase in AICD level when the EVs were treated with the  $\gamma$ -secretase inhibitor, L685,458 (Figure 2A,C).

In order to investigate whether A $\beta$  is generated in situ in EVs isolated from the brain of Tg2576 mice, we performed Western blotting experiments. We observed a decrease in the level of monomeric A $\beta$  (Figure 3A,B) and an increase in the level of A $\beta$  dimers (Figure 3A,C) following 24 hours of EV incubation. These findings were not affected by the treatment with the  $\gamma$ -secretase inhibitor, L685,458 (Figure 3A-C). While the SDS-PAGE band of ~8-9 kDa could derive from N-terminal extended (NTE) A $\beta$  forms,<sup>45,46</sup> the absence of an effect of the  $\gamma$ -secretase inhibitor on the levels of the ~8-9 kDa band (Figure 3A), excludes the possibility of NTE A $\beta$  generation during the incubation. These results suggest that EV-associated monomeric A $\beta$  was oligomerized into A $\beta$  dimers during the incubation. The level of A $\beta$  was also analyzed by sensitive ELISA measurements of A $\beta$  peptides in EVs incubated for 24 hours in DMEM. We found a robust decrease in the level of monomeric A $\beta$ 40 (Figure 3D), consistent with the Western blotting results. The level of A $\beta$ 42 was below the assay detection level (data not shown). The reduction in A $\beta$  level could be due to the activity of EV-associated A $\beta$ -degrading enzymes such as neprilysin,<sup>47</sup> insulin-degrading enzyme (IDE),<sup>48</sup> or endothelin-converting enzymes-1 and -2 (ECE-1 and ECE-2),<sup>49</sup> suggested to be active in the extracellular space.<sup>50</sup> Thus, we pretreated the brain EVs with the metalloprotease inhibitors phosphoramidon and thiorphan, in order to inhibit the activities of these enzymes. Treatment with these inhibitors did not affect the monomeric A $\beta$  levels (Figure 3E), suggesting that the decrease in A $\beta$  levels was not due to degradation by the metalloproteases against which inhibitors were used. We also found no effect of the inhibitors on the level of A $\beta$  dimers (Figure 3F). An increase in the ratio of A $\beta$  dimers to A $\beta$  monomers during EV incubation was observed in the absence and presence of the inhibitors (Figure 3G), suggesting a sustained A $\beta$  oligomerization during EVs incubation.

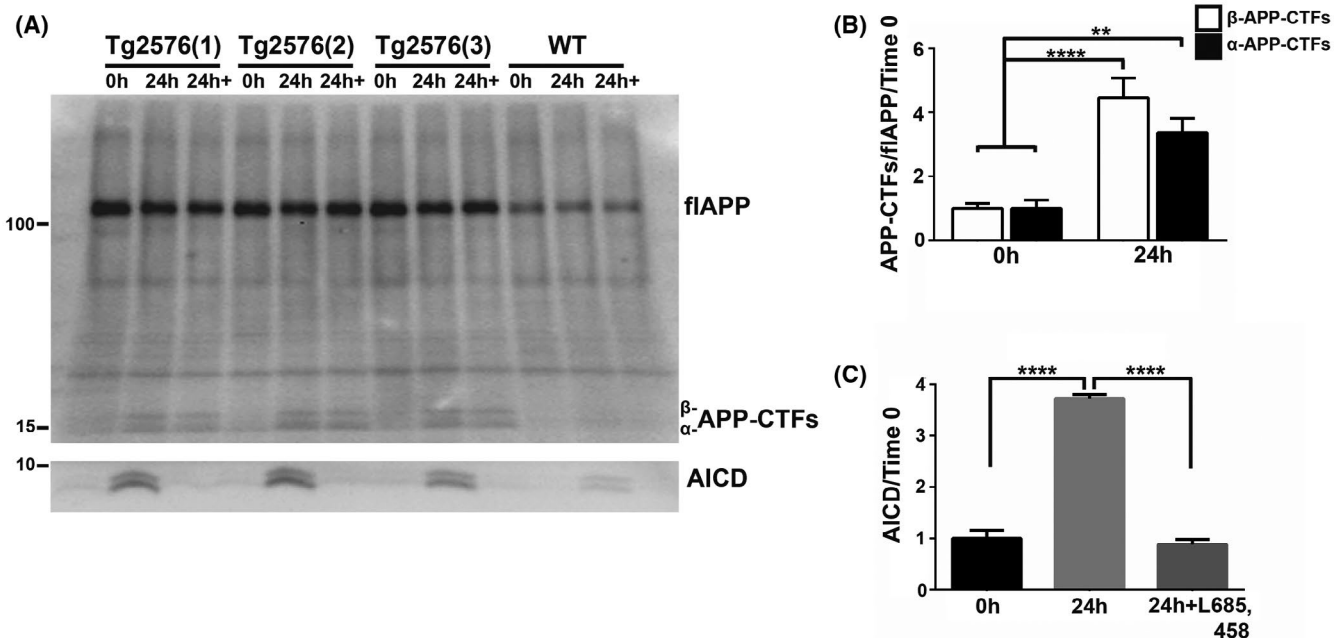
## 4 | DISCUSSION

We have previously reported that EVs are enriched with APP-CTFs.<sup>8,9</sup> Given that an early event that characterizes AD is a dysfunctional endosomal-lysosomal system (reviewed in 51), we proposed that the release of endosomal





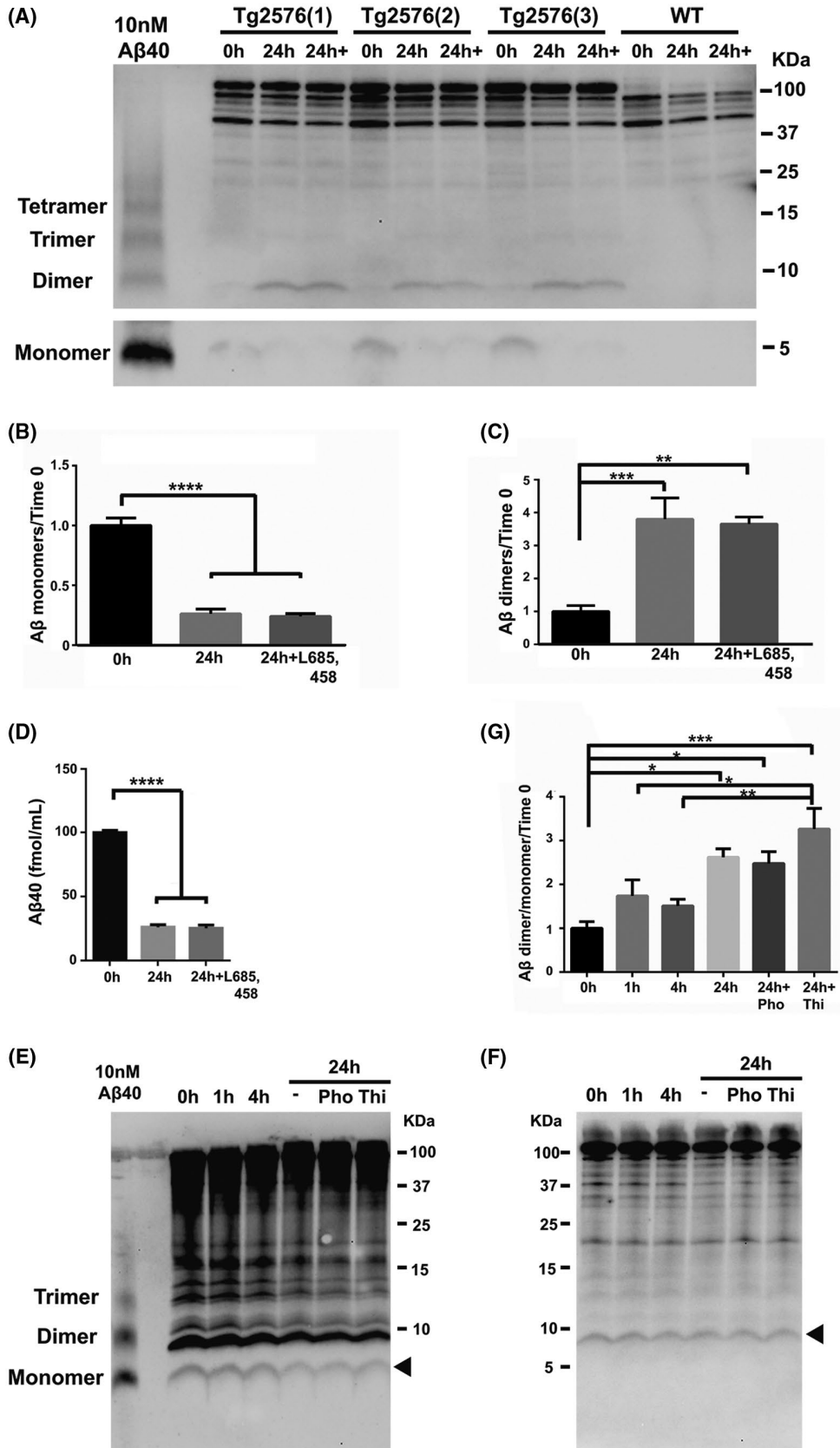
**FIGURE 1** flAPP is enzymatically processed in brain EVs to generate  $\alpha$ - and  $\beta$ -APP-CTFs in a time dependent manner. A, Western blot analyses revealing the presence of the APP processing enzymes ADAM10 ( $\alpha$ -secretase), BACE1 ( $\beta$ -secretase), and of all the components of the  $\gamma$ -secretase complex: PS1, PS2 APH1a, APH1b, Nicastrin, and PEN-2. Wild-type mouse brain homogenate (BH) was used as a positive control and arrow-heads indicate the specific protein band for each panel. B, Western blot analysis using an antibody against the carboxyl-terminus of APP (C1/6.1) showing an increase in the levels of  $\alpha$ - and  $\beta$ -APP-CTFs in wild-type brain EVs during incubation at 37°C for 24, 48, and 72 hours. Antibodies against the exosomal markers HSC70 and CD63 were used in the lower panels



**FIGURE 2** APP-CTFs in brain EVs are enzymatically cleaved by the  $\gamma$ -secretase complex, leading to the generation of AICD. A, Western blot analysis with the C1/6.1 antibody of EVs isolated from the brain of 4-month-old Tg2576 and wild-type (WT) mice prior to (0 hour) and after incubation at 37°C in DMEM without (24 hours) or with 10  $\mu$ M of the  $\gamma$ -secretase inhibitor L-685,458 (24 hours+). B, Quantification of  $\alpha$ - and  $\beta$ -APP-CTFs levels in EVs isolated from the brain of Tg2576 mice revealed an increase in the levels of both metabolites. C, The levels of AICD also increased and the increase was blocked by inhibition of the  $\gamma$ -secretase complex. PVDF membranes were used for APP and APP-CTFs detection, whereas nitrocellulose was used for detection of AICD. One-way ANOVA followed by Tukey's post hoc multiple comparison,  $n = 7$  independent experiments (B),  $n = 3$  independent experiments (C). Data are shown as mean  $\pm$  SEM (\*\* $P < .01$ ; \*\*\*\* $P < .0001$ )

materials into the extracellular space via EVs is a beneficial mechanism by which neurons remove accumulated materials, and that failure of efficient exosome production

and release can exacerbate<sup>7</sup> and perhaps lead to endosomal-lysosomal pathway disturbances.<sup>52</sup> Here, we demonstrate that APP-CTFs are generated by an active secretase



**FIGURE 3** A $\beta$  present at time zero in EVs oligomerizes into dimers. A, Western blot analysis of EVs isolated from the brain of 4-month-old Tg2576 and wild-type (WT) mice prior to (0 hour) and after incubation without (24 hours) or with 10  $\mu$ M of L-685,458 (24 hours+) shows the bands for A $\beta$  monomers (lower panel) and dimers. Quantification of the corresponding bands shows a decrease in the level of monomeric A $\beta$  (B) and an increase in the level of the dimers (C) following incubation. DMEM with 10 nM of A $\beta$ 40 peptide was used as a positive control of A $\beta$  monomeric and oligomeric forms. D, A $\beta$ 40 measurements of brain EVs by ELISA prior to (0 hour) or after a 24-hour incubation without (24 hours) or with L-685,458 (24 hours+). E and F, Western blot analysis of brain EVs after incubation for 1, 4, and 24 hours without (–) or with the metalloprotease inhibitors Phosphoramidon (Pho) or Thiorphan (Thi). A $\beta$  monomers (E) and dimers (F) are indicated by arrowheads. G, The protein bands were quantified in all conditions and presented as the ratio between A $\beta$  dimers and A $\beta$  monomers. Nitrocellulose membranes were used for detection of A $\beta$  monomers (E), whereas PVDF was used for detection of A $\beta$  dimers (F). One-way ANOVA followed by Tukey's post hoc multiple comparison,  $n = 3$  independent experiments (B),  $n = 7$  independent experiments (C),  $n = 5$  independent experiments (D), and  $n = 4$  independent experiments (G). Data are shown as mean  $\pm$  SEM (\* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .001$ ; \*\*\*\* $P < .0001$ )

machinery and accumulate in EVs over time. Therefore, EVs may contribute to the accumulation in the brain of  $\beta$ -APP-CTFs, suggested to be involved in the initiation of neurodegenerative processes associated with endosomal-lysosomal<sup>21,27,53</sup> and autophagy dysfunction,<sup>54,55</sup> neuroinflammation,<sup>28,53</sup> and synaptic alterations leading to cognition and memory deficits in AD.<sup>26,28,56</sup> Consistently, it was shown that the treatment with  $\gamma$ -secretase inhibitors leading to APP-CTFs accumulation impairs cognitive function in transgenic mice<sup>57</sup> and worsens cognitive deficits in AD patients.<sup>58</sup> Interestingly, inhibition of  $\gamma$ -secretase increased the levels of APP-CTFs and oligomeric APP-CTFs in EVs in an AD mouse model.<sup>59</sup> Here, we demonstrate that AICD, previously identified in EVs,<sup>11,17</sup> is also generated in brain EVs. Given the orientation of flAPP in the membrane, it is likely that AICD is released into and accumulates within EV lumen. It was demonstrated that in addition to AICD being a regulator of gene expression<sup>60</sup> and cell-signaling pathways,<sup>61</sup> it has deleterious effects such as causing hyperphosphorylation and age-dependent aggregation of tau, which recapitulates the features of AD,<sup>62</sup> and impairing hippocampal neurogenesis through the induction of neuroinflammation.<sup>63</sup> While we did not observe significant changes in the levels of APP-CTF when the EVs were incubated in the presence of a  $\gamma$ -secretase inhibitor, the decrease in the levels of AICD in the presence of the  $\gamma$ -secretase inhibitor indicates that  $\gamma$ -secretase is indeed active in EVs. This is likely due to the continuous cleavage of flAPP by  $\alpha$ - and  $\beta$ -secretases leading to the accumulation of  $\alpha$ - and  $\beta$ -APP-CTFs in the EV membrane and a low  $\gamma$ -secretase activity resulting in only a small part of APP-CTFs being further processed by  $\gamma$ -secretase.

Although less abundant than APP-CTFs, the peptide A $\beta$  has also been identified in association with EVs.<sup>9,16</sup> The association between A $\beta$  and EVs is supported by the findings of specific surface molecules in EVs that mediate their interaction with A $\beta$ , such as glycosphingolipids glycans,<sup>64</sup> ceramides,<sup>65</sup> and the GPI-anchored protein PrP<sup>c</sup>.<sup>66</sup> The interactions of A $\beta$  with PrP, glycosphingolipids, and ceramide localized in lipid raft domains may also target intracellular amyloids to EVs.<sup>67</sup> Regardless the A $\beta$  origin, it was

demonstrated that EVs promote A $\beta$  aggregation on their surface.<sup>33,34,65</sup> Since APP processing occurs in endosomes, EV-associated A $\beta$  may have an intracellular origin through its incorporation into intraluminal vesicles that will be released as exosomes upon fusion of the late endosome with the plasma membrane. While we detected an increase in the levels of APP-CTFs in EVs during ex vivo incubation, the levels of EV-associated monomeric A $\beta$  decreased dramatically. Although EVs have been implicated in the extracellular enzymatic degradation of A $\beta$ ,<sup>48,68</sup> the reduction in A $\beta$  levels that we observed was not due to active A $\beta$ -degrading metalloproteases present in EVs. However, we cannot rule out the possibility that other proteases present in EVs are mildly contributing to the degradation of A $\beta$ . The decrease in the levels of the monomeric A $\beta$  during the incubation was mostly associated with rapid assembly of the existing monomeric A $\beta$  into dimers, which was the most enriched A $\beta$  oligomer species in association with the EVs. Altogether, the data suggest that only negligible de novo generation of A $\beta$  occurs in EVs and that A $\beta$  associated with the EVs has mainly an intracellular origin. During incubation of the EVs, the A $\beta$  dimer/monomer ratio increased in a time-dependent manner. These results are consistent with previous reports showing that A $\beta$  oligomers are rapidly sequestered on membranes due to their low stability in the aqueous extracellular space.<sup>69</sup> The lower molecular weight A $\beta$  oligomers such as dimers have been suggested to be especially neurotoxic because they can act as seeds for further aggregation.<sup>70,71</sup> In addition, a recent study demonstrated that A $\beta$  dimers derived from AD brain mediated neuronal hyperactivity, which characterizes the early stage of AD, through block of synaptic glutamate reuptake.<sup>72</sup> The A $\beta$  dimers-enriched EVs may thus have a neurotoxic role at the early stages of AD by A $\beta$  oligomerization into dimers and we suggest that EV-associated A $\beta$  dimers could be used as a diagnostic marker to detect early AD.

More than a decade has passed since EVs were proposed to participate in the spread of A $\beta$  pathology in the AD brain.<sup>16</sup> Accordingly, it has been recently demonstrated that EVs are vehicles for the intercellular transmission of APP and APP metabolites,<sup>17</sup> including oligomeric A $\beta$ .<sup>73</sup> Our results support

that brain EVs are not only carriers, but also a site for the generation of APP-CTFs and AICD, and for the oligomerization of A $\beta$ , all neurotoxic peptides. Thus, EVs may propagate the disease within the brain by delivering toxic metabolites into recipient neurons. Indeed, it was shown that inhibition of EV secretion is protective by decreasing amyloid plaque deposition and ameliorating the cognitive impairment in transgenic APP mice.<sup>65</sup> However, apparently contradictory data suggest that EVs may act as potent scavengers for A $\beta$  in the brain<sup>64,74</sup> and contribute to the clearance of toxic proteins by microglia uptake<sup>33,75</sup> or by enzymatic degradation of A $\beta$ .<sup>76</sup> Whether EVs drive pathology spread or serve as a pathway for the removal of toxic molecules within the brain, as we have previously suggested,<sup>7,8</sup> is dependent on a balance between the release and efficient removal of EVs from the brain extracellular space. Given that the contribution of microglia to A $\beta$  clearance is reduced in advanced AD,<sup>77</sup> this balance is likely to tilt toward the pathological side of EVs as the disease progresses.

To conclude, our data along with a growing body of research suggest a working model where at early stages of AD, EVs have a protective role for cells in the brain. EVs are secreted loaded with APP and neurotoxic APP metabolites (APP-CTFs, AICD, and A $\beta$ ) to relieve neurons from accumulated toxic material. At that stage, EVs transport the material to either microglia for degradation, or out of the brain into the circulation. However, in advanced stages of AD with compromised endosomal-lysosomal and removal systems, EVs can be sites for the generation and accumulation of APP metabolites, which would potentiate AD-associated neurodegeneration. Therefore, determining the spatiotemporal functions of brain EVs in AD is crucial to further understand AD pathogenesis.

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## CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

## AUTHOR'S CONTRIBUTIONS

E. Levy and R. Pérez-González conceived the ideas and wrote and revised the manuscript. R. Pérez-González, Y. Kim, and E. Levy designed research. R. Pérez-González, Y. Kim, C. Miller, and J. Pacheco-Quinto performed experiments and analyzed data. R. Pérez-González, Y. Kim, J. Pacheco-Quinto, EA Eckman, and E. Levy interpreted results. All authors read and approved the final manuscript.

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