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Alterations in phosphatidylethanolamine levels affect the generation of Aβ

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Summary

Several studies suggest that the generation of Aβ is highly dependent on the levels of cholesterol within membranes' detergent-resistant microdomains (DRM). Indeed, the β-amyloid precursor protein (APP) cleaving machinery, namely β- and γ-secretases, has been shown to be present in DRM and its activity depends on membrane cholesterol levels. Counterintuitive to the localization of the cleavage machinery, the substrate, APP, localizes to membranes' detergent-soluble microdomains enriched in phospholipids (PL), indicating that Aβ generation is highly dependent on the capacity of enzyme and substrate to diffuse along the lateral plane of the membrane and therefore on the internal equilibrium of the different lipids of DRM and non-DRM domains. Here, we studied to which extent changes in the content of a main non-DRM lipid might affect the proteolytic processing of APP. As phosphatidylethanolamine (PE) accounts for the majority of PL, we focused on its impact on the regulation of APP proteolysis. In mammalian cells, siRNA-mediated knock-down of PE synthesis resulted in decreased Aβ owing to a dual effect: promoted α-secretase cleavage and decreased γ-secretase processing of APP. *In vivo*, in *Drosophila melanogaster,* genetic reduction in PL synthesis results in decreased γ-secretase-dependent cleavage of APP. These results suggest that modulation of the membrane-soluble domains could be a valuable alternative to reduce excessive Aβ generation.

Key words: alzheimer; amyloid; APP; secretase; lipids; phosphatidylethanolamine.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by numerous pathological features, including the presence of intracellular neurofibrillary tangles and extracellular β-amyloid plaques (Selkoe, 2001). Major components of the plaques are amyloid β-peptides (Aβ). Irrespective of the extent to which the plaques are end-points in the course of the majority of AD cases or truly causative, it is becoming more and more evident that soluble and short Aβ oligomers have a deleterious effect on neuronal function, eventually leading to cell death (Haass & Selkoe, 2007). Aβ is one of the cleavage by-products of the β-amyloid precursor protein (APP), resulting from the sequential proteolytic processing by β- and γ-secretases at the β- and γ-cleavage sites. In an alternative pathway, APP can be cleaved by α-secretase between the β- and γ-cleavage sites, producing a small non-toxic peptide (p3), thereby precluding the generation of Aβ (Selkoe, 2001). Because of the toxicity resulting from the excess of Aβ, it is of critical importance to understand how these alternative cleavages are regulated. Naturally, because APP and its proteases are all integral membrane proteins, the cleavage of APP will be greatly affected by changes in the cell's lipid content. Indeed, strong evidence indicates a functional relationship between AD and amyloidogenesis with lipid metabolism (Vetrivel & Thinakaran, 2006).

Several studies suggest that the generation of Aβ is highly dependent on the levels of cholesterol within membranes' detergent-resistant microdomains (DRM) (Cordy *et al.*, 2003; Vetrivel *et al.*, 2004). In fact, the APP cleaving machinery, namely β- and γ-secretases, has been shown to reside in DRM (Cordy *et al.*, 2003; Ehehalt *et al.*, 2003; Abad-Rodriguez *et al.*, 2004) and its activity depends on membrane cholesterol levels (Ehehalt *et al.*, 2003; Grimm *et al.*, 2008). Interestingly, although many have found that overexpressed APP in certain cell lines is retained within DRM (c.f.e.g. (Ehehalt *et al.*, 2003), at constitutive levels of expression in neuronal cells APP is localized in detergent-soluble microdomains (or non-DRM), membrane domains enriched in phospholipids [PL; (Abad-Rodriguez *et al.*, 2004; Vetrivel *et al.*, 2005)]. Although abnormal brain PL metabolism has been implicated in the pathogenesis of AD, the data are correlative and in many instances contradictory, making it difficult to draw conclusions about the true implication of these lipids in pathology. For example, phospholipid levels were found to be decreased in frontal lobe white matter and hippocampus from brains of patients with AD (Svennerholm & Gottfries, 1994), yet phosphatidylserine was found to be increased in synaptosomes prepared from affected regions of AD brains (Wells *et al.*, 1995). Of the PLs, PE membrane levels are affected in AD brains, while phosphatidylcholine (PC) and phosphatidylinositol (PI) do not show significant changes (Wells *et al.*, 1995; Prasad *et al.*, 1998).

Together with PC, PE is the most abundant PL in eukaryotic cells constituting 20-40% and 55% of the total PLs in mammalian and in *Drosophila melanogaster* cell membranes, respectively. PE is involved in a variety of cell processes. It has been shown to be required for membrane protein activity; it serves as 'chaperone' and, along with other 'non-bilayer' lipids, PE held to be important for maintaining the physical state of the bilayer (Dowhan & Bogdanov, 2009). Unlike PC and PS, which form flat bilayers, PE can also form non-bilayer structures under physiological conditions. These structures, observed *in vitro*, may provide discontinuity in the membrane bilayer for several important biological functions such as vesicle-mediated protein trafficking, lateral movement of macromolecules and stabilization of specific membrane protein complexes (Dowhan & Bogdanov, 2009). PE is synthesized via two main pathways: CDP-ethanolamine pathway (Kennedy, 1957), Fig. 1A: Kennedy pathway) and phosphatidylserine (PS) decarboxylation pathway (Merrill *et al.*, 1997). Because the CDP-ethanolamine pathway is the major source of PE in insects (Downer, 1985) and in mammalian tissues such as

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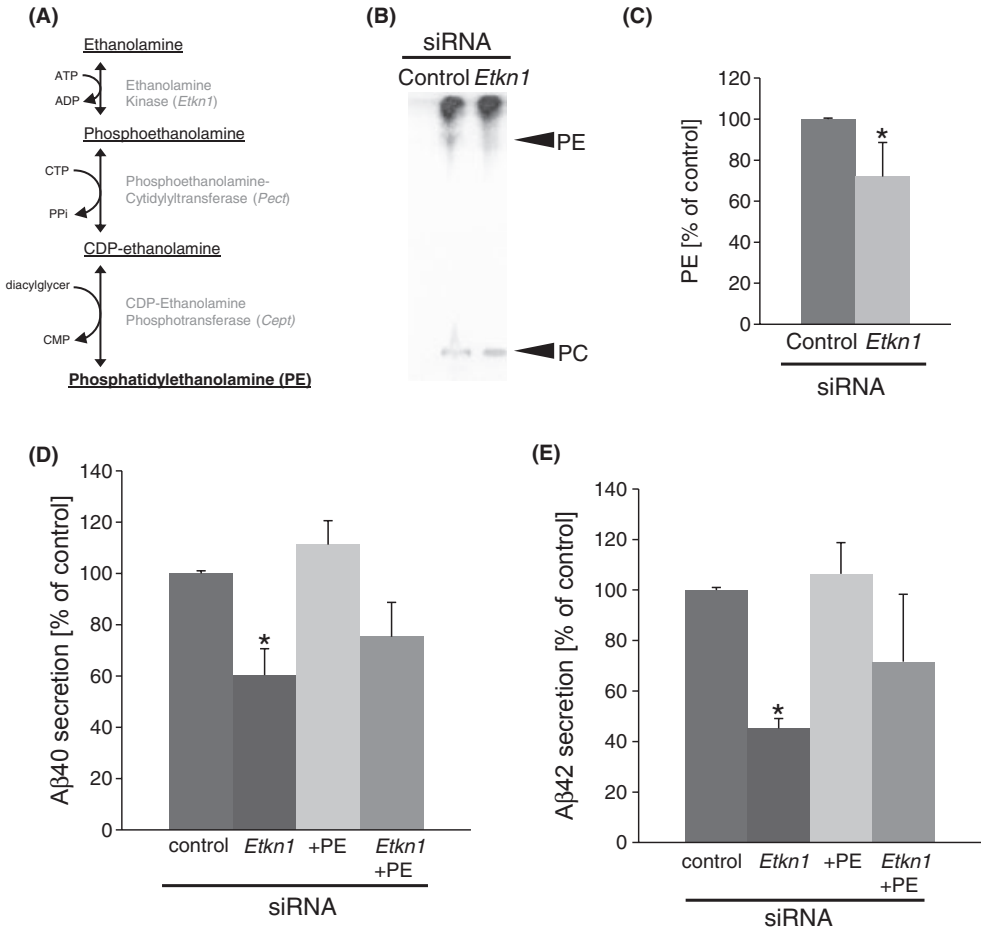

Fig. 1 Depletion of PE decreases Aβ secretion in mammalian cells. (A) Scheme displaying the PE synthesis pathway. (B) HEK293 cells overexpressing APP695 were treated with siRNA against ethanolamine kinase 1 (*Etkn1*). 48 h post-transfection, cell membranes were isolated and membrane lipids were extracted. Efficiency of the siRNA reduction of the candidate gene was analysed by TLC. (C) PE levels were normalized against the total amount of PC and displayed as percentage of control. Values represent means of three independent experiments ± SD. Significance was determined by Student's *t*-test (**P* value: 0.014). (D–E) Media of HEK293 cells overexpressing APP695 and treated with non-targeting (control) or specific siRNA against *Etkn1* were collected 72 h post-transfection. Another set of cells was treated with PE (0.001%) for 12 h. Secreted Aβ40 and Aβ42 load was evaluated by ELISA. Values represent means of three independent experiments ± SD each. Significance was determined by Student's *t*-test **P* values are *Etkn1*: 0.0005, *Etkn1* + PE: 0.13 and +*PE*: 0.054 for Aβ40 and *Etkn1*: 0.0001, *Etkn1* + PE: 0.14 and +*PE*: 0.4 for Aβ42. Absolute Aβ40/Aβ42 levels (pg mL^{-1}) of a representative experiment are control: 393/110, *Etkn1*: 190/50, *Etkn1* + PE: 236/58 and +*PE*: 455/137.

the brain and the heart (Arthur & Page, 1991), we concentrated our efforts in the study of this pathway.

Here, we have shown that PE levels play an important role in Aβ generation. In mammalian cells, siRNA-mediated knock-down of PE synthesis resulted in decreased Aβ generation caused by a lowered γ-secretase processing and a promoted α-secretase cleavage of APP. In *Drosophila melanogaster,* genetic reduction in PE resulted in decreased γ-secretase-dependent cleavage of APP. As PE reduction did not alter membrane cholesterol content, nor affected the expression of other membrane lipids, we have reached the conclusion that the tight regulation of the lipidic environment around APP in non-DRM is an important parameter for the regulation of APP proteolysis and thus Aβ generation.

Results

Decreased Aβ secretion in mammalian cells with reduced levels of *Etkn1*

To determine to which extent depletion of PE affects the cleavage of APP in mammalian cells, we knocked down the mammalian ethanolaminekinase1 (*Etkn1)* (see Fig. 1A) by sequence-specific siRNAs in HEK293 cells stably expressing human APP (APP695). We also depleted the phosphoethanolamine-cytidyltransferase (*Pect*) and CDP-ethanolamine-phosphotransferase (*Cept*); these two enzymes, involved in PE-synthesis, act downstream of *Etkn1* in the CDP-ethanolamine pathway (see Fig. S1A). siRNA efficacy was tested after 72 h by measuring membrane PE levels in siRNA-treated vs. non-treated (control) cells (Figs 1B and S1A). As further control, cells were treated with siRNA directed against an irrelevant messenger RNA (rat *Cyp7a1*) (Fig. S1A). Cell membrane pellet isolation, followed by lipid extraction, lipid separation by Thin layer chromatography (TLC) and lipid measurement, revealed a clear and significant (30%) reduction in PE levels in *Etkn1* siRNA-treated cells compared with the non-treated cells (Fig. 1B–C), whereas the membrane content of cholesterol, ceramide, sphingomyelin, PC and PI was not significantly affected by treatment at the analysed time-points (Fig. S1A–C). Also, treatment with the control *Cyp7a1* siRNA had no effect on membrane lipid content (Fig. S1A,D). To test whether siRNA treatment and PE depletion have a deleterious effect on the cells, cell viability was assayed. Apoptosis levels, measured by TUNNEL assay, revealed no significant difference in cell viability between siRNA-treated/PE-depleted and control cells (data not shown).

First, we measured Aβ levels in the medium of *Pect*, *Cept* and *Etkn1* knock-down cells. ELISA-based analysis of Aβ levels in the medium at 72 h post-transfection revealed a significant decrease in Aβ40, up to 40% (Figs 1D and Fig. S2B). Likewise, the secretion of Aβ42 was significantly decreased in *Pect*-, *Cept*- and *Etkn1*-depleted cells compared with controls (up to 50%, Figs 1E and S2C). Contrary, exogenous addition of PE to the cells not significantly but slightly increased the secretion of Aβ. Moreover, exogenous addition of PE to *Etkn1*-depleted cells partially reversed the phenotype (Fig. 1D–E). Second, to further validate this, we proceeded to analyse the amount of Aβ by peptide immunoprecipitation/Western blot. The data depicted in Fig. S2A confirm that siRNA knock-down of *Pect*, *Cept* and *Etkn1* reduced the amount of secreted Aβ. Given that knock-down of all three enzymes involved in the CDP-

ethanolamine pathway had a similar effect on PE content and Aβ secretion, the remainder of this work is focused on the effect of inhibiting the *Etkn1* enzyme, which mediates the first step in the PE synthesis.

Depletion of PE promotes α-secretase-mediated cleavage of APP in mammalian cells

The observation that PE depletion resulted in reduced Aβ production led us to investigate its effect on activities of the different secretases involved in APP cleavage. Membrane preparations, from non-treated (control) and *Etkn1* siRNA-treated HEK293 cells stably overexpressing human APP695, were analysed for the different APP processing products by Western blotting. In siRNA-treated cells, with reduced *Etkn1* expression (Fig. 2A) and around 30% less membrane PE (see Fig. 1C), the CTFβ/α ratio was about 30% lower compared with cells with normal PE levels (Fig. 2B,D). Consistent with this last result, the ratio APPsβ/α in *Etkn1* siRNA-treated cells was about 50% lower (Fig. 2E–F) in comparison with the control cells, indicating an increased α-secretase processing or decreased β-secretase cleavage of APP (Figs 2B–F and S2D–F). To assess whether the changes in the generation of CTFα and CTFβ were attributable to the alterations in APP expression, the levels of full-length APP were determined in low PE vs. control cells (Figs 2B–C and S2D–F). Quantification of APP normalized to the total amount of tubulin revealed the absence of any evident alteration in APP content (Figs 2C and S2E). Furthermore, siRNA-mediated knock-down of *Pect* and *Cept* had similar effects on APP cleavage (Fig. S2D–E). As control, cells were treated with siRNA directed against *Cyp7a1*. This treatment affected neither APPsα, CTFα and CTFβ production nor APP expression (Fig. S2D,F). In agreement with previous results, the effect on Aβ production by increasing phosphatidylethanolamine could not be evaluated, owing to the apoptotic effect of increasing this lipid (Singh *et al.*, 1996).

To further demonstrate the impact of PE depletion on APP cleavage, HEK293 cells stably expressing APP695 were treated for 72 h with the siRNA against *Etkn1*, radiolabelled with [^{35}S] methionine, and APP and its proteolytic processing derivatives were immunoprecipitated from cell lysates and conditioned media, utilizing antibodies directed against APP intracellular and extracellular domains, respectively. Compared with cells overexpressing APP alone, cells co-expressing APP and *Etkn1* siRNA presented a considerable higher level of CTFα (Fig. 2G), indicating an increased α-secretase processing of APP. In this line, the Aβ secretion also appeared decreased in PE-depleted cells (Fig. 2G). The protein levels of ADAM9, ADAM10 and ADAM17, member of the α-secretase family, or

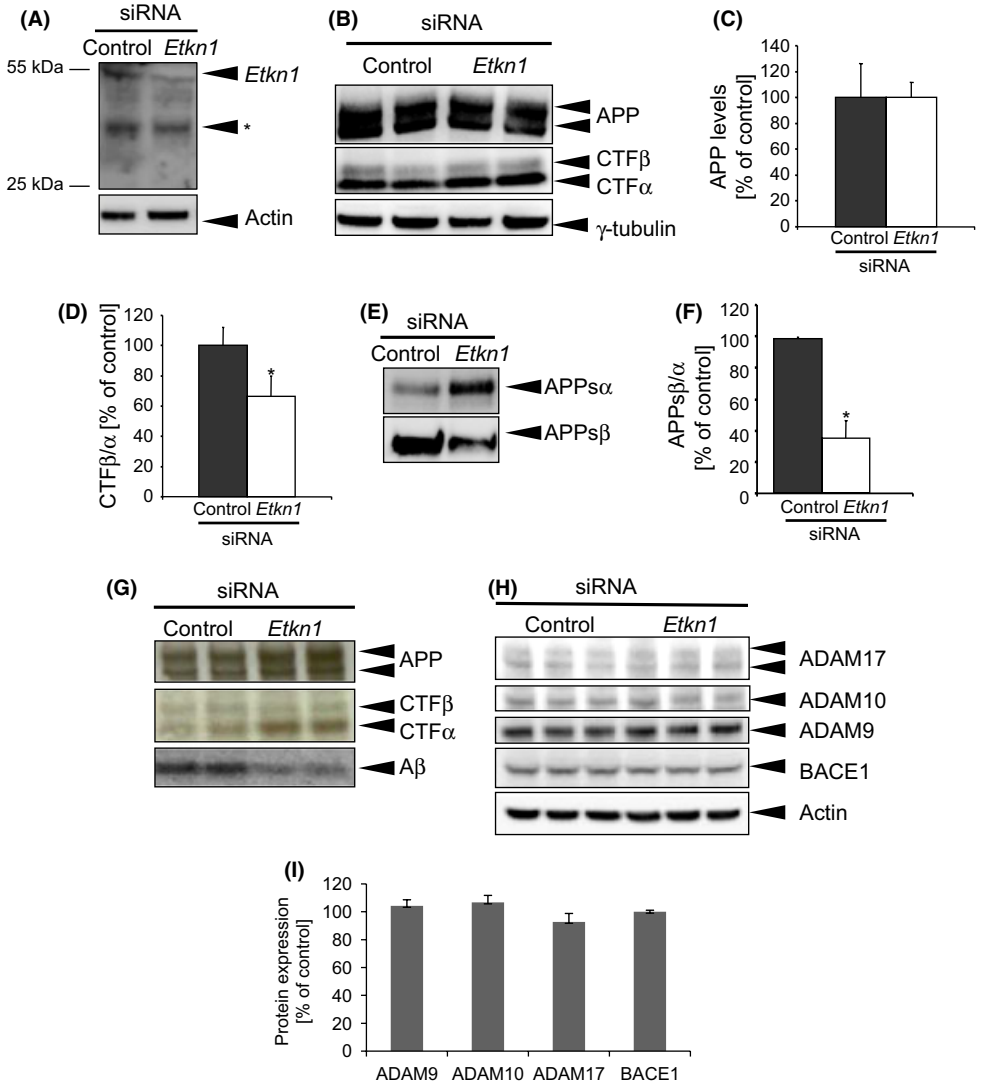

Fig. 2 Depletion of PE synthesis affects APP processing. (A–B) For the determination of APP processing products, crude membrane pellets isolated from non-treated (control) and *Etkn1* siRNA-treated HEK293 cells were subjected to SDS-PAGE and analysed by Western blot. *Etkn1* level in the treated cells was determined using *Etkn1* antibody (*unspecific band). Levels of APP, CTFα and CTFβ were detected using APPCt antibody. γ-Tubulin served as a loading control. (C) Protein levels of APP are displayed as percentage of control (n = 3). (D) The CTFβ/α ratio (n = 3, *P value = 0.007). (E) APPsα and APPsβ were detected in conditioned media using antibody 6E10 that selectively detects APPsα and 39138 specific for Appsβ. (F) The APPsβ/α ratio (n = 3, *P value = 0.001). (G) HEK293 cells expressing APP695 alone were transfected with non-targeting (control) or *Etkn1* siRNA. 72 h post-transfection, the cells were pulse labelled with [^{35}S]methionine for 2 h. Cells were chased for 4 h, and secreted APP was immunoprecipitated with APP NT antibody that recognizes both APPsα and APPsβ, or immunoprecipitated with antibody 6E10 that selectively detects APPsα and Aβ. APP and CTFs were immunoprecipitated with APPCt antibody from cell lysates. Precipitates were separated by SDS-PAGE, and radiolabelled proteins were detected by phosphoimaging. (H) Lysates from HEK293 cells overexpressing APP695 and transfected with the candidate siRNA were analysed for the expression levels of ADAM9, ADAM10, ADAM17 and BACE1. Quantifications in (I) represent the mean ± SD of three independent experiments.

of the β-secretase (BACE1) were not altered (Fig. 2G–I), indicating that PE depletion does not have a direct effect on the expression of the two enzymes. From this set of data, we can conclude that depletion of PE promotes α-secretase cleavage of APP. Even though the use of clonal cell lines overexpressing human APP is a widely accepted tool to study APP processing, additional work is required to determine whether the changes reported here occur also when the endogenous protein is studied.

Depletion of PE decreases γ-secretase-mediated cleavage of APP in mammalian cells

To investigate the role of PE on γ-secretase cleavage of APP, the generation of the γ-secretase cleavage product APP intracellular domain (AICD) was analysed in an *in vitro* assay (Sastre, 2010). Depletion of PE led to a clear reduction in γ-secretase-mediated cleavage of APP, measured by AICD levels (Fig. 3A). Addition of PE to the *in vitro* assay increased the generation of AICD and was even able to rescue the decreased γ-secretase activity in PE-depleted cells (Fig. 3A).

An important aspect of the γ-secretase activity is substrate promiscuity. The list of proteins processed by the γ-secretase complex is constantly growing, yet the one considered most crucial, together with APP, is the Notch receptor. Notch is a type-I transmembrane protein, which follows similar sequential proteolysis to that of APP. Cleavage of Notch by the γ-secretase complex leads to the generation of Notch intracellular domain (NICD), which translocates to the nucleus where it acts as a regulator of transcription (De Strooper *et al.*, 1999; Struhl & Greenwald, 1999). Notch intracellular domain-dependent Notch signalling pathway controls

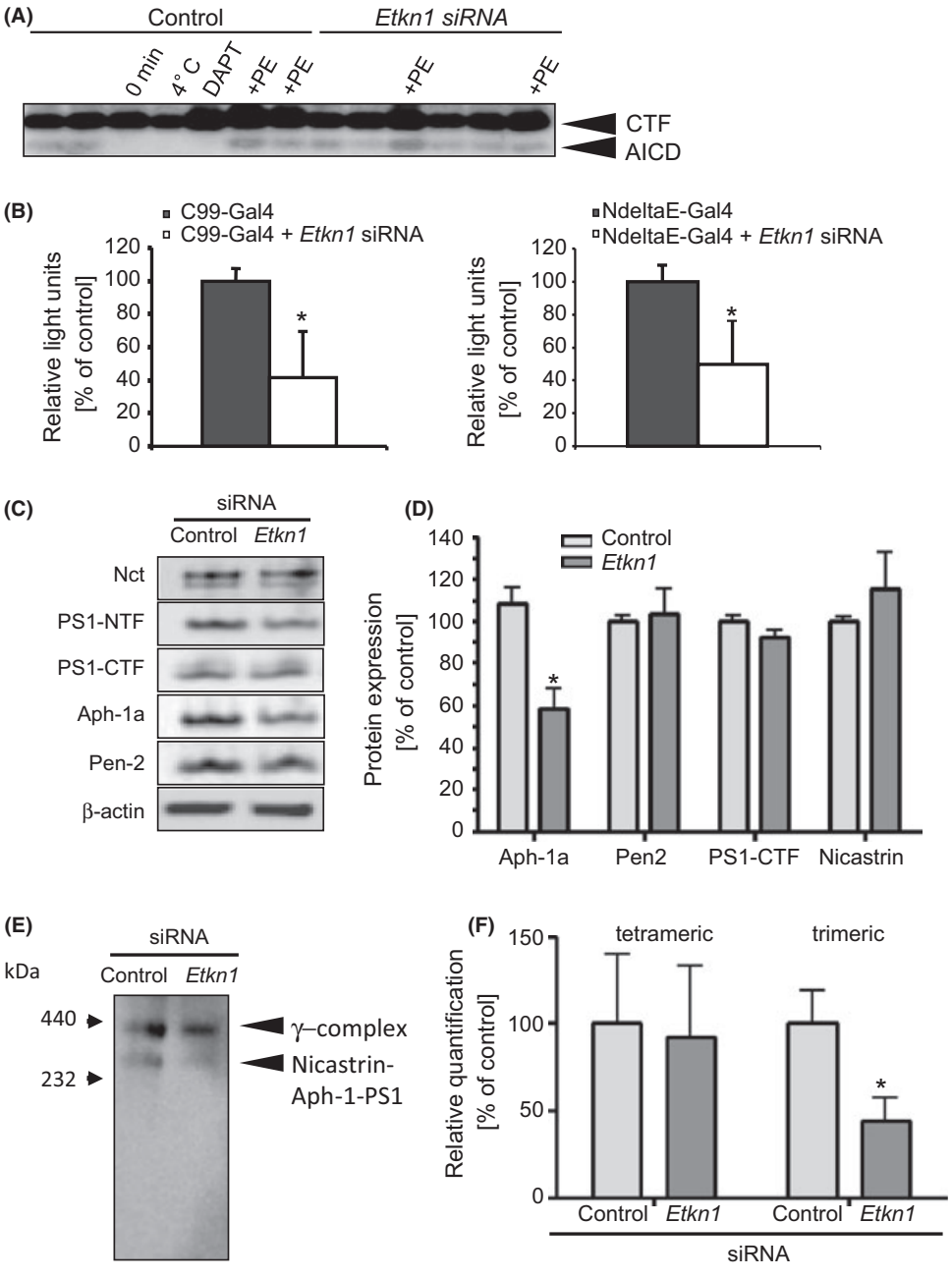

Fig. 3 Suppression of PE synthesis decreases γ-secretase cleavage. (A) *In vitro* γ-secretase assay: cell membranes from HEK293-expressing APP695 transfected with *Etkn1* or control siRNA were prepared. The isolated membrane fractions were resuspended in citrate buffer and incubated at 37 °C for 2 h. The generation of the γ-secretase cleavage product AICD was analysed by Western blot using APPCt antibody. Incubation for 0 min at 4 °C to inhibit enzymatic activity or in the presence of the γ-secretase inhibitor DAPT (1 μM) was used as negative control. Note that addition of PE (0.001%) to the *in vitro* assay rescues the γ-secretase activity in membranes of *Etkn1*-depleted cells. (B) HEK293 cells were transfected with C99-Gal4-VP16, NotchΔE-Gal4-VP16, or Gal4-V16 and UAS-luciferase and co-transfected with the siRNA collection. The luciferase activities of the γ-secretase-dependent variants were determined: C99-Gal4-VP16 (n = 5; *P value 0.001) and NotchE-Gal4-VP16 (n = 5; *P value 0.002). Values are presented as means ± SD, and significance was assessed using Student's t-test. (C) Lysates from HEK293 cells overexpressing APP695 and transfected with the candidate siRNAs were analysed for the expression levels of the components of γ-secretase, namely presenilin (PS1-NTF and CTF), nicastrin (Nct), Aph-1 and Pen-2. β-actin was used as loading control. Note that Nct appears as double band corresponding to the mature (m) and immature (im) protein. (D) Values represent gene expression normalized to β-actin as means of three assays ± SD (Aph-1a: *P value = 0.01). (E) Cell membranes of HEK293 expressing APP695 transfected with *Etkn1* or control siRNA were processed for BN-PAGE. Western blot analysis of nicastrin using 9C3 antibody indicates that down-regulation of *Etkn1* results in relatively decreased levels of tetrameric mature complexes and significant lower levels in the intermediate trimeric (Nct–Aph1-PS1) subcomplex. (F) Relative quantification represented as means of three assays ± SD (trimeric complex: *P value = 0.03).

embryonic cell-fate decisions in variety of cell lineages (Struhl & Adachi, 2000).

To determine the impact of PE levels on γ-cleavage of Notch, we analysed the levels of AICD and NICD production in HEK293 cells expressing APPC99-GAL4/UAS-luciferase or NotchΔE-GAL4/UAS-luciferase reporter constructs. HEK cells were co-transfected with the reporter constructs and *Etkn1* siRNA oligonucleotides; AICD or NICD generation was monitored after 72 h. The knock-down of *Etkn1* resulted in a 50% decreased AICD generation (Fig. 3B, left panel). NICD generation was also significantly reduced by 50% (Fig. 3B, right panel), indicating that PE is required for the proper formation or activity of the γ-complex.

In line with this possibility, we firstly investigated the expression levels of the four components namely presenilin (PS 1/2), nicastrin (Nct), Pen-2 and Aph-1. We detected a significant decrease in the amount of Aph-1a, while the expression of PS1 was unaffected and the amount of Nct and Pen-2 was only slightly increased (Fig. 3C–D). Secondly, we determined the γ-complex formation using blue native PAGE (Bammens *et al.*, 2011). Using this technique, we observed relatively lower levels of mature complex assembly and a significant decrease in the formation of the intermediate Nct–Aph-1-PS1 subcomplex in *Etkn1*-depleted cells (Fig. 3E–F).

Together, we could show that depletion of PE affects the activity of the γ-secretase, probably via affecting the expression or stability of Aph-1a and therefore the correct assembly of the mature γ-complex.

Depletion of PE decreases γ-secretase-mediated APP processing in transgenic *Drosophila in vivo*

Modelling of human neurodegenerative diseases in *Drosophila melanogaster* is a strategy that has been widely adopted and has undeniably improved the knowledge of mechanisms involved in the pathogenesis of Alzheimer's disease (Bilen & Bonini, 2005). Drosophila endogenously expresses orthologues to all four components of the γ-secretase complex and offers a highly conserved γ-secretase activity, making it a suitable model to investigate γ-secretase cleavage of APP (Bilen & Bonini, 2005).

To gain insight into the possible role of PE in APP processing *in vivo*, we analysed the effect of a mutation in the *Etkn* gene in *Drosophila melanogaster*. Beside sterols and sphingolipids, PLs constitute the main lipid species in *Drosophila* membranes (Jones *et al.*, 1992). Barring differences in the length of fatty acyl chains, PLs in mammals and *Drosophila* share a similar organization. PE comprises 55% of total PLs in *Drosophila* (Jones *et al.*, 1992) and is synthesized via the CDP-ethanolamine pathway (Downer, 1985). *Etkn* has been located on the gene *easily shocked* (*eas*), on the X chromosome. *EasPC80* mutants, defective in *eas* gene, lack the highly conserved kinase domain, which is required for *Etkn* enzymatic activity (Pavlidis *et al.*, 1994). In accordance with *Etkn* loss of function, *easPC80* mutants showed a decreased PE level compared with the wild-type flies (Fig. S3A). *EasPC80* homozygous flies are viable and, under most conditions, do not show any observable abnormalities, regarding development, viability, behaviour or electrophysiology (Pavlidis *et al.*, 1994).

To determine the effect of PE depletion on APP processing by γ-secretase in *Drosophila*, *easPC80* mutants were crossed with GMR > C99-GAL4/UAS-GRIM transgenic flies, which act as a reporter of γ-secretase activity in the fly eye (Guo *et al.*, 2003). Briefly, γ-secretase substrate is obtained by the presence of the chimeric type-I protein containing the human CTFβ (C99) transmembrane protein fragment of APP fused to the yeast transcription factor GAL4. C99-GAL4 is specifically expressed in the eye under control of the eye-specific GMR promoter. The reporter flies additionally carry a γ-secretase reporter output construct, the *Drosophila* cell-death reporter gene GRIM, cloned under the UAS sequence. After γ-secretase cleavage, C99-C-terminal fragment translocates together with GAL4 to the nucleus and triggers the transcription of the cell lethal gene GRIM, through UAS activation (Guo *et al.*, 2003 and Fig. 4A for representation). Activation of GRIM transcription promotes cell death in the fly eye, so that the size of the eye and the roughened surface correlate with the level of endogenous γ-secretase activity acting on the transgenic reporter. Control flies, containing the reporter construct transgenic for GMR > C99-GAL4/UAS-GRIM but with a wild-type membrane PE level, showed a certain level of retinal cell death owing to the normal levels of endogenous γ-secretase activity. Approximately 70% of total eye surface was roughened in the γ-reporter control flies, and the eye size was, compared with the wild-type eye, about 20% decreased (Fig. 4B panels i and ii). *EasPC80f* homozygous males and females, carrying GMR > C99-GAL4/UAS-GRIM, were then compared with these control flies. Retinal cell death, which was present in the control flies, was almost eliminated, and the eye size was restored to normal by introducing *easPC80f* mutation, corresponding to decreased γ-secretase activity and less expression of the cell lethal gene (Fig. 4B panels iii and iv). Membrane PE levels in *easPC80* homozygous flies, expressing GMR-GMR > C99-GAL4/UAS-GRIM, were reduced up to 50% compared with wild-type flies, while the PE levels in γ-secretase reporter flies did not differ from those found in the wild-type (Fig. S3B–C).

Because the GMR promoter was expressed during eye formation, we decided to rule out whether the reduced γ-cleavage might indicate a function of PE during development. To determine whether PE depletion affects γ-cleavage in the adult flies, we produced a fly line with the C99-GAL4-VP16 (GV) construct under a heat-shock promoter (hs). These hs > C99-GV flies were combined with the UAS-cd8-GFP reporter gene, which allowed us to detect GFP expression upon γ-secretase activity (hs > C99-GV/UAS-cd8-GFP). These reporter flies were then crossed with *easPC80* homozygous females. Male flies were selected from the progeny, collected during 5 days heat-shocked for 1 h at 38 degrees and scored 24 h later for GFP expression. These flies showed a 50–60% decrease in membrane PE levels compared with wild-type flies (Fig. S3B,D). As shown in Fig. 4C,D, depletion of PE decreased GFP expression significantly.

To exclude non-specific effects on reporter gene expression (expression independent from cleavage-mediated UAS activation), *easPC80* homozygous flies were combined with the GMR-GAL4/UAS-G/RPR reported flies that do not contain the C99 chimeric protein. Because in this case GAL4 is not fused to C99, and not under the control of γ-secretase, a true modifier of γ-secretase should induce cell death in the retina of the reporter flies containing C99 but not in the ones that lack C99. Depletion of PE alone had no effect on GRIM expression (cell death), indicating a specific effect of *easPC80* mutation through lowering PE levels on γ-secretase activity (Fig. 4E). In addition, membrane PE levels were determined in GMR-GAL4, UAS-G/RPR *easPC80* homozygous vs. GMR-GAL4, UAS-G/RPR wild-type flies. TLC analysis of membrane lipid composition showed that *easPC80f* mutants had about 50% lower membrane PE levels than the wild-type flies. Ergosterol, ceramide, PECeramide, PC and PI membrane levels were in contrast not significantly different in mutant and wild-type flies (Fig. 4F).

Discussion

As one would expect, lipids are potent modulators of APP processing: cholesterol, sphingolipids, ceramides and PLs were reported to correlate with AD pathogenesis (Prasad *et al.*, 1998; Han, 2005). Among PLs, particularly PE membrane levels are affected in AD brains (Wells *et al.*, 1995; Prasad *et al.*, 1998). Although it remains to be determined whether these changes are at the base of the disease (i.e. leading to abnormal

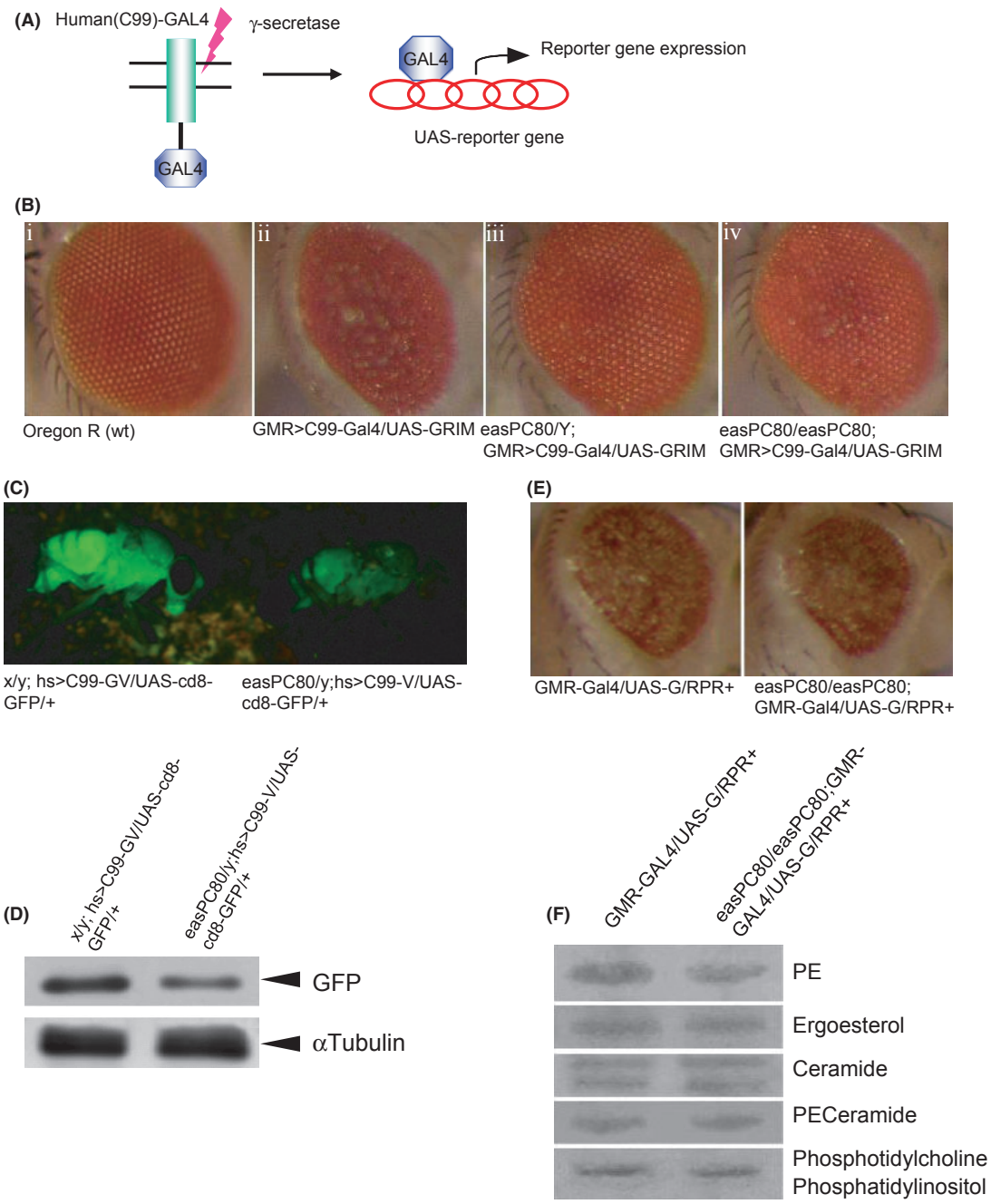

Fig. 4 PE depletion decreases APP processing *in vivo*. (A) The *Drosophila* read-out system: The γ-secretase reporter contains two components: the chimeric protein APPC99-Gal4 that serves as a γ-secretase substrate and the UAS-reporter gene construct as an output. In the absence of γ-secretase activity, APPC99-GAL4 remains attached to the membrane and is disabled to enter the nucleus and activate the transcription of the reporter gene. In the presence of γ-secretase activity, cleavage of APP releases the C-terminal part of APPC99 that is fused to the Gal4 sequence. This fragment translocates to the nucleus and activates reporter gene transcription. (B) Light microscope images of adult fly eyes of various genotypes: Oregon R (wild-type) (i), GMR > C99-GAL4/UAS-GRIM (ii), *easPC80*/Y; GMR > C99-GAL4/UAS-GRIM (males; iii) and the *easPC80*/*easPC80*; GMR > C99-GAL4/UAS-GRIM (females, iv). γ-secretase activity was measured by cell death levels in control reporter flies (GMR > C99-GAL4/UAS-GRIM; ii) vs. reporter flies homozygous for *easPC80* mutation (iii, iv). Retinal cell death was scored by quantification of eye size and the size of roughened eye surface. (C) GFP signal in *Drosophila* transgenic flies with following genotypes: x/y; hs > C99-GV/UAS-cd8-GFP/+ and *easPC80*/y; hs > C99-GV/UAS-cd8-GFP/+ was measured 12 h after heat-shock-induced activation of the C99-GV construct. GFP expression measured by fluorescence intensity in the entire fly shows a decrease of about 65% in mutants with respect to control flies. (D) An additional analysis of GFP expression by Western blot analysis revealed, similar to the fluorescence intensity measurements, an approximately 65% lower GFP level in *easPC80* mutants vs. control reporter flies. Tubulin served as the loading control. (E) Constitutively expressed G/RPR, in GMR-GAL4, UAS-G/RPR flies that do not contain the human C99 protein fragment, leads to caspase-dependent cell death *easPC80* mutants expressing GMR-GAL4, UAS-G/RPR/+ constructs genotype: *easPC80*/*easPC80*; GMR-GAL4, UAS-G/RPR/+ revealed that the size of roughened eye surface and the eye size do not differ in mutants and control flies. (F) Membrane lipids extracted from the membranes of 20 fly heads were analysed by TLC and subsequently quantified for PE, ergosterol, ceramide, PECeramide, PC and PI amount in *easPC80* homozygous expressing GMR-GAL4, UAS-G/RPR/+ vs. GMR-GAL4, UASG/RPR/+ flies without *easPC80* mutation (control).

production of Aβ or inhibiting Aβ degradation) or are simple side-effect of the numerous changes that occur in its course, our work suggests that reduction in neuronal PE slows down Aβ production. Additionally, our study demonstrates that this is at least owing to a dual process: promotion of α-secretase cleavage and decrease in γ-secretase-mediated processing of APP (Fig. 5). While the second seems to be a direct effect on enzymes' activity, as demonstrated in this study, the first could be a more indirect effect, probably due to altered accessibility of the substrate to the enzyme. Yet, we cannot rule out additional mechanism involved in (i.e. increased degradation of Aβ).

PE depletion promotes α-secretase cleavage of APP

As mentioned beforehand, a possible explanation for the increased levels of α-secretase APP cleavage products and concomitant decreased β-secretase processing of APP is the separation of the cleaving machinery and the substrate into distinct membrane microdomains. Growing evidence indicates the importance of DRM that are enriched in active β- and γ-secretases, as principal membrane platforms for amyloidogenic processing of APP (Ehehalt *et al.*, 2003; Abad-Rodriguez *et al.*, 2004; Vetrivel *et al.*, 2004). On the contrary, α-secretase has been shown to act in the non-DRM (Kojro *et al.*, 2001; Ehehalt *et al.*, 2003). As APP at its constitutive levels of expression is mainly restricted to the non-DRM of neuronal and non-neuronal cells (Abad-Rodriguez *et al.*, 2004; Vetrivel *et al.*, 2005), Aβ can only arise by mechanisms that allow the lateral diffusion of β- and γ-complex away from DRM or of APP towards DRM. Our results indicate that PE content is crucial for this diffusion: cleavage of APP by β- and γ-secretases decreases after PE depletion. The elevated levels of α-secretase products in low PE cells might therefore be explained by the retention of APP in PL-rich domains, where α-secretase resides. This possibility is illustrated in Fig. 5B.

PE depletion prevents γ-cleavage of APP

Because γ-secretase is a four-protein complex with 19 transmembrane domains and its active site is predicted to be near the centre of the bilayer, it is likely that its conformation and activity will be highly sensitive to changes in membrane lipid composition. Reconstitution of γ-secretase in complex lipid mixtures revealed that, although all subcellular membrane lipid mixtures supported robust γ-cleavage activity, a DRM-like composition supported the highest level of activity compared with other membrane compositions (Osenkowski *et al.*, 2008). These results suggest that DRM do not only bring the key players in Aβ production, i.e. β-secretase, APP and γ-secretase together, but also that the lipidic environment of the DRM has the ability to support the highest γ-secretase activity, thereby promoting Aβ production (Ehehalt *et al.*, 2003; Vetrivel *et al.*, 2004). On the other hand, semi-purified and purified γ-secretase preparations have been found to be inactive unless the main lipids of non-DRM, PC and PE are added to the reaction mixture (Fraering *et al.*, 2004; Wrigley *et al.*, 2005), highlighting the importance of non-DRM to support activity. Our results clearly demonstrate that PE content is crucial for APP processing and unequivocally show that depletion of PE decreases the activity of the γ-secretase complex. This is caused by alterations in the expression or stability of Aph-1 and therefore the correct assembly of the mature γ-complex (Fig. 5C). It has been shown that the four core components of the γ-secretase complex tightly regulate each other's expression, maturation and assembly (Edbauer *et al.*, 2002; Takasugi *et al.*, 2003). The earliest step in complex assembly is probably the association of Nct with Aph-1. Full-length PS might bind to Nct-Aph-1 precomplex. This is followed by recruitment of Pen-2 (Spasic & Annaert, 2008). In our study, we found that reduction in PE significantly alters the protein level of Aph-1 and thus reduces the correct assembly of the precomplexes. The physiological role of Aph-1 is still to be determined (Steiner *et al.*, 2008) but it is important for assembly, stabilization and trafficking of the mature γ-secretase complex (Takasugi *et al.*, 2003; Pardossi-Piquard *et al.*, 2009). Aph-1 exists as two homologues, Aph-1a and Aph-1b, and the two different Aph-1 proteins alter the conformation of the catalytic subunit PS in the complex as assessed by fluorescent lifetime imaging microscopy (Serneels *et al.*, 2009), suggesting that they have a structural effect on the catalytic site of the γ-secretase complex. In addition, it has been reported that inactivation of the Aph-1b gene leads to improvements of

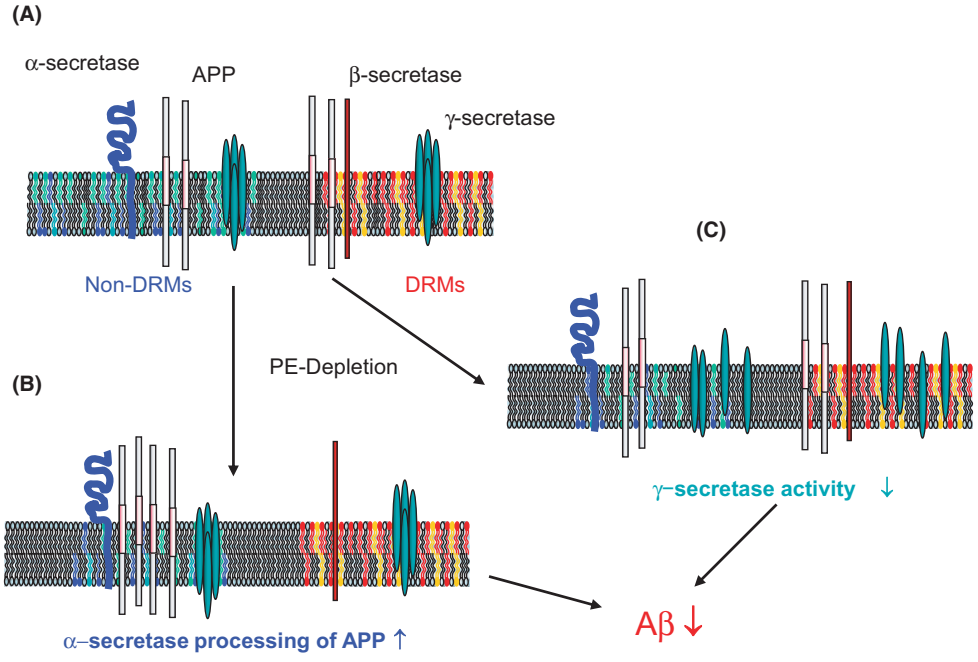

Fig. 5 Model showing the role of PE in the proteolytic processing of APP. (A) Contrary to β- and γ-secretase activity in DRM, α-secretase involved in non-amyloidogenic APP processing resides outside DRM. It seems likely that PL-rich membrane subdomains (non-DRM) together with DRM control the access of APP to its processing enzymes. (B–C) Depletion of PE affects the proteolytic processing at least in two distinct ways: via promoting the cleavage of APP by α-secretase (B) and by affecting the activity of γ-secretase (C). Perturbed membrane PL content could disable the access of β-secretase to APP. On the contrary, PE depletion could increase the accessibility of APP to α-secretase, illustrated by more APP molecules in the surrounding of α-secretase, both resulting in increased α-cleavage of APP (B). Furthermore, PE depletion leads to decreased γ-cleavage of APP, possibly by destabilizing the complex formation/stability, depicted by dissociated γ-complexes, and therefore γ-secretase activity (C).

AD-relevant phenotypic events such as amyloid plaque load (Serneels *et al.*, 2009).

Indeed, reduced PE levels could have affected the levels of Aph-1 via changes in protein folding. PE was shown to be involved in the correct folding and assembly of membrane proteins and membrane protein complexes (Bogdanov *et al.*, 1999). The most compelling evidence for a specific role of PLs in membrane protein folding is the necessity for PE to be present in the folding of the integral membrane protein lactose permease (LacY) of *Escherichia coli* (Bogdanov & Dowhan, 1995; Bogdanov *et al.*, 1996). LacY is organized within the inner cytoplasmic membrane as 12 transmembrane domains. Normal assembly of LacY occurs into *E. coli* membranes containing an abundance of PE. In contrast, depletion of PE leads to partial misfolding of the protein combined with partial loss of activity of LacY (Bogdanov *et al.*, 2008). Thus, one could envision that PE also facilitates the folding of proteins of the γ-complex into a fully native conformation by interacting with late folding and non-native intermediates, as do most conventional protein molecular chaperones. Naturally, upon a reduction in PE levels, proper protein folding would not occur, the proper assembly of the γ-complex will be at least partially abolished, and consequently, γ-activity will be also reduced (Fig. 5C). In line with this, we observe in the present study lower levels of mature γ-complex as well as precomplex formation under PE depletion. This is an interesting venue of future investigation and could lead to the discovery of therapeutic targets with far more restricted effect than the reduction in a lipid.

In conclusion, this work expands our view on the role of membrane lipids in relation to Aβ generation by showing that PE, a lipid highly enriched in the environment where APP naturally resides, plays an important role in the regulation of APP proteolysis and thus Aβ generation.

Materials and methods

Drosophila genetics and maintenance

All stocks were maintained at 25 °C and raised on cornmeal-yeast agar medium. The fly reporter C99-GAL4/UAS-GRIM and hs > C99-GV flies have been previously described (Struhl & Adachi, 2000; Guo *et al.*, 2003). *EasPC80*f mutant flies were a kind gift of Dr Mark Tanouye.

Mammalian cell lines and primary rat hippocampal neurons

Human embryonic kidney (HEK) 293 cells stably overexpressing human wild-type APP695 were grown in Dulbecco's modified Eagle's minimum (DMEM) essential medium (GIBCO, Merelbeke, Belgium) supplemented with 10% heat-inactivated foetal bovine serum (Perbio, Erembodegem, Belgium) and 100 μg mL^{-1} penicillin/streptomycin (GIBCO). Cell lines were kept under 5% CO2 at 37 °C.

Antibodies

For immunoprecipitation, uptake assays, cell surface labelling experiments and detection of Aβ and secreted APPα, the 6E10 antibody (Sigma, Bornem, Belgium) or for Aβ alone 4G8 (Sigma) was used. Secreted APPβ was detected using 39138 antibody (Convance, Princeton, NJ, USA). APP C-terminal fragments as well as AICD were precipitated with APPCt C-terminal antibody (Sigma). Total secreted APP and APP full length were detected with the APPNt N-terminal antibody (Sigma). Presenilin was detected with the SB129 antibody, nicastrin with the 9C3 antibody (Esselens *et al.*, 2004), Aph-1a with the B80.3 (Nyabi *et al.*, 2003) and Pen-2 (Nyabi *et al.*, 2003) with the B126.2 antibody. γ-Tubulin and β-actin antibody were purchased from Sigma. *Etkn1* antibody was obtained from Abcam (Cambridge, UK).

siRNA design and transfection

The siRNA sequences for rat and human were designed using an RNAi algorithm publicly available at http://www.dharmacon.com. For our assays, 100 pmol siRNA was transfected per well in a 6-well dish using the Lipofectamine 2000 (Invitrogen, Merelbeke, Belgium) transfection reagent, according to the manufacturer's instructions.

ELISA

For Aβ peptide detection in HEK293 cells, the cells were transfected with siRNA and the medium was replaced with fresh 48 h post-transfection. Cells were grown for another 24 h, and media were assayed for Aβ load using the hAβ40 or hAβ42 ELISA kit (The Genetics Company, Basel, Switzerland) according to the manufacturer's instructions.

Immunoprecipitation and immunoblotting

Cells were lysed 72 h post-transfection in STEN lysis buffer (1 × STEN: 50 mM Tris pH 7.6, 150 mM NaCl, 2 mM EDTA, 0.2% NP40; STEN-lysis buffer: 1% Triton X100, 1% NP40, complete protease inhibitors in 1 × STEN) and clarified by a 30-min centrifugation at 16 000 *g*. For immunoprecipitation of proteins, the lysates or the conditioned media were incubated overnight with the appropriate antibodies and protein G sepharose beads (Amersham, Diegem, Belgium). The beads were washed twice with STEN-NaCl (STEN buffer with 500 mM NaCl) and once with STEN buffer. Upon SDS-PAGE electrophoresis, immunoprecipitated proteins were transferred to nitrocellulose membrane and detected with the corresponding antibodies.

Metabolic labelling

72 h post-siRNA transfection, HEK293 stably overexpressing APP695 were starved for 45 min and labelled with radioactive methionine/cysteine [^{35}S] for 2 h. For the proteolytic products of APP, the cells were chased for 4 h and lysates and media were analysed for CTF generation and APP secretion, respectively. Radiolabelled proteins were visualized with phosphoimaging.

In vitro γ-secretase assays

HEK293 APP695 cells were transfected with the siRNAs, and 48 h post-transfection cell membranes were prepared. Cells were harvested and resuspended in hypotonic buffer (10 mM Tris, pH 7.3, 10 mM $MgCl_2$, 1 mM EDTA and 1 mM EGTA) for 10 min on ice. Cells were then homogenized by passing 15 times through a 21-gauge needle and centrifuged for 10 min at 100 *g* to pellet nuclei. The resulting supernatant was centrifuged 30 min at 16 000 *g*. Pelleted membranes were resuspended in citrate buffer (150 mM sodium citrate, adjust pH 6.4 with citric acid, complete protease inhibitors) and incubated at 4 °C (as negative control) or at 37 °C for 2 h. Analysis of γ-secretase products was performed with standard SDS-PAGE.

γ-secretase luciferase assay

For the assay, HEK293 cells were co-transfected with the UAS-luciferase reporter gene, an APP or Notch reporter construct carrying a Gal4-VP16

(Serneels *et al.*, 2005) in the cytoplasmic domain, and specific siRNA oligonucleotides targeting the candidate genes. After 48 h, the cells were lysed and processed according to the manufacturer's instructions (Promega, Leiden, Netherlands), and emitted light was measured with the microplate reader (Victor3 by PerkinElmer, Zavetem, Belgium).

Blue-native PAGE

Cell membranes were prepared as described above and resuspended in buffer containing 0.5% dodecylmaltoside, 20% glycerol, 25 mM Bis–Tris/HCl, pH 7.0, and protease inhibitors and solubilized for 1 h on ice. After ultracentrifugation (100 000 *g* at 4 °C for 30 and 15 min), the cleared supernatant was collected. For each sample, the same amount was mixed with 5 × BN sample buffer (2.5% Coomassie brilliant blue G-250, 50 mM Bis–Tris/HCl, 250 mM 6-amino-caproic acid, pH 7.0, and 15% sucrose) and stored overnight at 4 °C. BN-PAGE was performed as described previously (Bammens *et al.*, 2011) with some modifications. Samples were loaded on a 5–16% polyacrylamide gel and electrophoresed at 200 V for 4 h. Before transference, the gel was treated with transfer buffer containing 0.1% SDS for 10 min.

Thin layer chromatography

Mammalian cell lines

Cell membranes obtained after 100 000 *g* centrifugation were resuspended in 0.9% (w/v) NaCl containing 37% (v/v) HCl and homogenized with a 22-gauge syringe. Membrane lipids were than extracted according to Bligh and Dyer (1959). After addition of 2 mL methanol and 1 mL chloroform to 800 μL suspension containing cell membranes, 1 mL chloroform and 1 mL of 0.9% (w/v) NaCl containing 37% (v/v) HCl were added to the sample, mixed well, incubated 10 min on ice to allow the phase separation and centrifuged at 100 *g* for 5 min at 4 °C. The upper phase was discarded and 2 mL methanol plus 1.8 mL of 0.9% (w/v) NaCl/37% (v/v) HCl were added to the interphase and the lower phase and kept on ice for minutes. Subsequently, the samples were centrifuged at 100 *g* for 5 min at 4 °C, and the lower phase was collected and dried in a rotational vacuum concentrator (Centrifuge RVC 2-25; Christ) to obtain lipid pellets. Lipid pellets were resuspended in 1:1 methanol/chloroform mixture, applied to the silica gel 60 HPTLC plates (Merck, Overijse, Belgium) and separated first in the hydrophilic running solvent, composed of chloroform/acetone/acetic acid/methanol/H_2O (50:20:10:10:5) and subsequently in the hydrophobic running solvent containing hexane/ethyl acetate (5:2). The HPTLC plates were than dried at room temperature and stained with 7% (v/v) H_2SO_4 in methanol. Standards for cholesterol, ergosterol and sphingomyeline (Sigma), and PC, PE and PECeramide (Matreya, Pleasant Gap, PA, USA) were used to identify these lipid species. For the lipid quantification, the scanned TLCs were analysed in conditions of non-saturated signal by IMAGE J software.

Drosophila

For the membrane lipid analysis, 10 fly bodies (GFP reporter flies) or 20 fly heads (GRIM reported flies) were homogenized in lysis buffer [10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10% glycerol, 50 mM NaF, 1 mM NaVOF, 5 mM DTT, 4 M urea, and protease inhibitors (CLAP)]. After homogenization, the fly debris were centrifuged for 10 min by 18 000 *g* at 4 °C, followed by the protein measurement in supernatant and cell membrane centrifugation by 100 000 *g* at 4 °C for 1 h, using the same amount of total protein as starting material for all samples. Subsequently, the lipids were extracted and analysed as described above.

Statistical analysis

Quantifications were performed from at least three experimental groups. Data are presented as mean ± SD. *P* values were determined by Student's two-tailed *t*-test between control and experimental groups.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 Depletion of PE synthesis.

Fig. S2 Depletion of PE synthesis affects APP processing.

Fig. S3 Membrane PE content in *Drosophila* with different genotypes.

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