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Effects of Membrane Lipids on the Activity and Processivity of Purified γ-Secretase

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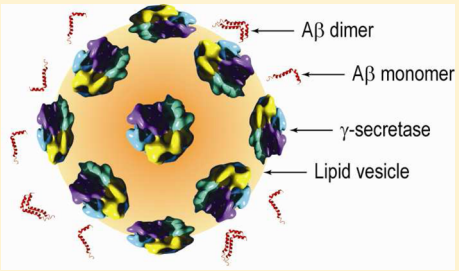
Supporting Information

ABSTRACT: The 19-transmembrane multisubunit γ-secretase complex generates the amyloid β-peptide (Aβ) of Alzheimer's disease (AD) by intramembrane proteolysis of the β-amyloid precursor protein (APP). Despite substantial advances in elucidating how this protein complex functions, the effect of the local membrane lipid microenvironment on γ-secretase cleavage of substrates is still poorly understood. Using detergent-free proteoliposomes to reconstitute purified human γ-secretase, we examined the effects of fatty acyl (FA) chain length, saturation and double-bond isomerization, and membrane lipid polar headgroups on γ-secretase function. We analyzed γ-secretase activity and processivity [i.e., sequential cleavages in the APP transmembrane domain that convert longer Aβ species (e.g., $Aβ_{46}$) into shorter ones (e.g., $Aβ_{40}$)] by quantifying the APP intracellular domain (AICD) and various Aβ peptides, including via a bicine/urea gel system that detects multiple Aβ lengths. These assays revealed several trends. (1) Switching from a *cis* to a *trans* isomer of a monounsaturated FA chain in phosphatidylcholine (PC) increased γ-activity, did not affect $Aβ_{42}$:$Aβ_{40}$ ratios, but decreased the ratio of long (≥42) versus short (≤41) Aβ peptides. (2) Increasing the FA carbon chain length (14, 16, 18, and 20) increased γ-activity, reduced longer Aβ species, and reduced the $Aβ_{42}$:$Aβ_{40}$ ratio. (3) Shifting the position of the double bond in 18:1(Δ9-*cis*) PC to the Δ6 position substantially reduced activity. (4) Gangliosides increased γ-activity but decreased processivity, thus elevating the $Aβ_{42}$:$Aβ_{40}$ ratio. (5) Phosphatidylserine decreased γ-activity but increased processivity. (6) Phosphatidylinositol strongly inhibited γ-activity. Overall, our results show that subtle changes in membrane lipid composition can greatly influence γ-secretase activity and processivity, suggesting that relatively small changes in lipid membrane composition may affect the risk of AD at least as much as presenilin or APP mutations do.



Alzheimer's disease (AD) is a neurodegenerative disorder defined by abundant intracellular neurofibrillary tangles of the tau protein and extracellular plaques of the amyloid β-protein (Aβ), leading to progressive loss of memory and cognition.[1] The β-amyloid precursor protein (APP) can be processed through either an amyloidogenic or nonamyloidogenic pathway. The former is initiated by β-secretase cleavage of holoAPP, leading to shedding of the ectodomain (APPs-β) and a 99-residue C-terminal fragment (C99) remaining embedded within the membrane.[2,3] In contrast, nonamyloidogenic processing occurs when the initial ectodomain shedding is caused by α-secretase, leading to release of a slightly longer ectodomain (APPs-α) and leaving a membrane-bound 83-residue stub (C83).[4] Subsequent intramembrane proteolysis of C99 or C83 by γ-secretase leads to the release of the APP intracellular domain (AICD) and either Aβ peptides[5] or p3 peptides,[6] respectively. γ-Secretase is a 19-transmembrane domain aspartyl protease comprised of presenilin (PS1 or PS2 isoform), nicastrin (Nct), anterior pharynx defective-1 (Aph1αL, Aph1αS, or Aph1β isoform), and presenilin enhancer-2 (Pen-2), which are necessary and sufficient for γ-activity.[7−9] γ-Secretase is responsible for the second and final step in regulated intramembrane proteolysis (RIP) of a large and increasing number of substrates, including APP and Notch.[10] A further complexity of γ-secretase function is that cleavage of the membrane-anchored C99 occurs at multiple sequential peptide bonds, starting with ε-cleavage to release AICD from the membrane, leaving a 49- or 48-residue Aβ. Sequential cleavages of $Aβ_{48/49}$ every three or four residues moving N-terminally occurs first at the so-called ζ-site to produce $Aβ_{45/46}$, then at the γ-site to produce $Aβ_{42/43}$, and finally at the γ′ site to produce $Aβ_{38/40}$ peptides.[11] $Aβ_{42}$ is generally considered the most pathogenic Aβ species, with an elevated $Aβ_{42}$:$Aβ_{40}$ ratio used as a marker of pathogenicity, although recently, $Aβ_{43}$ has also been shown to be pathogenically relevant in vivo.[12] The degree to which the initial ε-cleavage products $Aβ_{48}$ and $Aβ_{49}$ are trimmed by γ-secretase to shorter Aβ peptides is termed processivity.[13] Much work has focused on the understanding of how mutations,[13−16] protein cofactors,[17,18] and small organic compounds[19,20] can modify γ-secretase processing of APP, but far less on how the local membrane lipid environment could also affect function.

Evidence suggests that lipid composition is altered in AD brain tissue, but whether this is a cause or effect or both is unclear.[21,22] Most studies of effects of lipids on γ-secretase

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function have focused on cholesterol, including its high concentration in detergent-resistant membrane microdomains (DRMs),[23,24] where APP, BACE1, and γ-secretase can all be found (reviewed in ref 25). Moreover, epidemiological evidence suggests that cholesterol-lowering drugs (statins) may reduce AD risk, but whether statins can be used to prevent or treat AD is controversial.[26] In addition to cholesterol, there are a large number of other lipid types present in membranes.[27]

Bilayer-forming lipids can differ in various attributes, including fatty acyl (FA) chain length, level, position and type of unsaturation, and membrane lipid polar headgroup type. FA chain length has direct effects on membrane fluidity and thickness, the latter of which could affect the $A\beta_{40}$:$A\beta_{42}$ ratio.[28] With the advent of food processing over the past century, the modern human diet now contains elevated levels of *trans* isomer fatty acids, which have been linked with an increased risk of coronary heart disease.[29] Whether such "*trans* fats" may also increase the risk of AD is not clear. *Trans* fats have been reported to enhance amyloidogenic and inhibit nonamyloidogenic processing of APP in vitro and ex vivo,[30] and some evidence suggests an elevated risk of AD with a high level of dietary saturated and *trans* unsaturated fats.[31] Another report suggested no association between high intake of *trans* unsaturated fats, cholesterol, or other fats and an increased risk of developing AD.[32]

The direct effects of membrane lipid composition on the processing of APP by γ-secretase have been studied very little, and clarifying this issue may suggest the involvement of particular lipids in AD pathogenesis as well as guide dietary and therapeutic strategies for reducing AD risk. We recently developed a method for reconstituting purified human γ-secretase complexes into lipid vesicles with a defined composition.[33] Using this system, we have systematically tested the effects of membrane bilayer composition on Aβ generation, varying certain specific features, including FA chain length, the degree, position, and type of FA chain unsaturation, and the membrane lipid polar headgroup. We report here that bilayer composition can have profound effects on the production of Aβ, the $A\beta_{42}$:$A\beta_{40}$ ratio, and the processive trimming of long Aβ peptides to shorter, secreted forms.

EXPERIMENTAL PROCEDURES

Reagents. All lipids were from Avanti Polar Lipids. POPC, SOPC, and specific FA chain-containing PC are semisynthetic. L-α-Phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE), L-α-phosphatidylserine (PS), total ganglioside extract (GS), sphingomyelin (SM), and whole brain lipid extract (WB) are from porcine brain. L-α-Phosphatidylinositol (PI) was from bovine liver and L-α-phosphatidic acid (PA) from chicken egg.

Purification of γ-Secretase and the Recombinant Substrate. High-grade purification of human γ-secretase from our S20 cell line (coexpressing untagged human PS1 and human Nct-V5/His, Aph1αL-HA, and FLAG-Pen-2) was performed by a multistep protocol.[34] Purification of recombinant C100-FLAG was performed as described previously.[35]

Reconstitution of γ-Secretase To Form Proteoliposomes. Proteoliposome preparation was performed as described previously[33] with the following modifications. (1) Hydrated lipids and lipid mixtures were diluted to a total concentration of 1.5 mM in 50 mM HEPES (pH 7.2), 150 mM NaCl, 4 mM CHAPSO, and purified γ-secretase. (2) Detergent was removed (and proteoliposome formation) when the samples were incubation with SM-2 BioBeads (Bio-Rad) at a concentration of 10 mg/mg of CHAPSO for 2 h at 4 °C and mixed with a HulaMixer (Life Technologies). Vesicle formation was checked by negative-stain electron microscopy as described previously.[33]

In Vitro γ-Secretase Activity Assays. Detergent-free γ-secretase proteoliposomes were incubated with 1 μM C100-FLAG at 37 °C for 4 h. Samples were assayed by Western blot for AICD, for $A\beta_{40}$ and $A\beta_{42}$ by ELISA (Life Technologies), or by bicine/urea Western blot for Aβ peptides, as described previously.[13]

Cell Treatment Experiments. CHO-derived S20 cells were seeded at a density of 5×10^5 cells/well in six-well plates (Falcon) growing in a DMEM/10% FBS mixture (Gibco). Twenty-four hours after the initial seeding, hydrated and sonicated lipids (or HEPES buffer alone as a blank control) were added to a final concentration of 10 or 50 μM. Twenty-four hours later, cells were washed twice with 2 mL of PBS, and then fresh medium and lipids were added. A further 24 h later, media were harvested and cells lysed with RIPA buffer and protease inhibitor cocktail (Roche). Protein concentrations of the clarified lysates were measured by a BCA assay (Thermo Scientific).

Western Blotting, ELISAs, and Antibodies. AICD Western blot analysis was performed by electrophoresing activity assay samples on a 4 to 12% Bis/Tris polyacrylamide gel, transferring them to a polyvinylidene difluoride membrane, and probing them with anti-human nicastrin (1:1000; BD Transduction Laboratories), anti-PS1-NTF (1:1000; Calbiochem), and anti-FLAG M2 (1:2000; Sigma). All Western blots were scanned on an Odyssey Infrared Imaging System (Li-Cor), and densitometry analysis used Odyssey software. $A\beta_{40}$ and $A\beta_{42}$ from in vitro activity assays were measured with an ELISA (Invitrogen). Lipid-treated conditioned media were assayed for $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ using a triplex ELISA (Aβ capture by antibody 4G8) read on a Sector Imager 2400 (Mesoscale Discovery).

Statistical Analysis. All quantified data represent an average of at least three independent experiments. Error bars represent the standard error. Comparison between samples was analyzed by one-way ANOVA with a post-test Bonferroni correction.

RESULTS

Because cleavage of substrates by the γ-secretase complex occurs within their transmembrane domains (TMDs) and γ-secretase itself has 19 TMDs and a membrane-embedded active site, the composition of the lipid bilayer intimately surrounding these proteins would be expected to have significant effects on proteolytic processing. With this in mind, we performed activity assays on purified γ-secretase[34] reconstituted into detergent-free lipid vesicles having systematic changes in membrane composition. Using methods modified from our previous membrane lipid study,[33] we generated unilamellar proteoliposomes with a mean diameter of 230 nm (95% confidence interval of 207−252 nm; $n = 94$) as measured by negative-stain electron microscopy (Figure 1B). Without this detergent removal step, we observed lipid/detergent layers in irregular shapes (Figure 1A), with γ-secretase activity unaffected by different FA chain lengths and degrees of saturation (data not shown).

Previous studies of the effects of the membrane lipid microenvironment have looked at certain polar headgroups,[33,36,37] but there has been only very limited analysis of

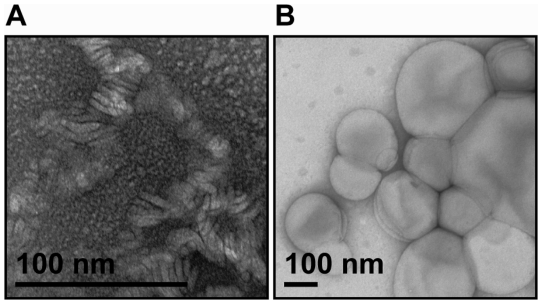

Figure 1. Effect of removal of detergent on proteoliposome morphology. Lipid, CHAPSO, and purified γ-secretase complexes were incubated together to form proteoliposomes and then observed by negative-stain electron microscopy (A) before or (B) after removal of the detergent with SM2 BioBead treatment at 4 °C for 2 h. Note the difference in magnification between the two images because of the smaller size of the structures without detergent removal.

the effects of FA chains.[30] With a change from *cis* to *trans* isomers of monounsaturated FA chains, the membrane density and fluidity should be altered because of changes in hydrophobic tail packing.[27,38] With a *cis* isomer double bond, the FA chain has a kinked orientation, resulting in a less densely packed bilayer and consequently increased fluidity, whereas a *trans* isomer of the same FA chain length will be more densely packed and have a higher phase transition temperature (i.e., the membrane is less fluid).[39] Given that the 19-TMD γ-secretase complex and its substrates are always embedded in membranes, they need to move within the lipid bilayer to allow substrate docking, lateral gating, cleavage, and release of products. As a result, membrane fluidity could affect substrate recognition, handling, and proteolysis. In the first half of this report, we describe the effects of FA chain length and isomerization (*cis* and *trans*) with phosphatidylcholine headgroups on γ-secretase activity and processivity (see Table 1 for a summary of the fatty acyl chains that were examined). In the second half, we investigate the effects of varying polar headgroups of membrane lipids.

Table 1. Descriptive and Common Names for the Fatty Acyl (FA) Side Chains Used with a Phosphocholine Headgroup[a]

descriptive name	common name
14:1 (Δ9-*cis*)	myristeleic acid
14:1 (Δ9-*trans*)	myristelaidic acid
16:0/18:1 (Δ9-*cis*)	palmitic/oleic acid (POPC)
16:1 (Δ9-*cis*)	palmitoleic acid
16:1 (Δ9-*trans*)	palmitelaidic acid
18:0/18:1 (Δ9-*cis*)	stearic/oleic acid (SOPC)
18:1 (Δ6-*cis*)	petroselenic acid
18:1 (Δ9-*cis*)	oleic acid
18:1 (Δ9-*trans*)	elaidic acid
18:2 (Δ9,12-*cis*)	linoleic acid
20:1 (Δ11-*cis*)	eicosenoic acid
20:4 (Δ5,8,11,14-*cis*)	arachidonic acid (AA)
22:1 (Δ13-*cis*)	erucic acid
22:6 (Δ4,7,10,13,16,19-*cis*)	docosahexaenoic acid (DHA)

[a]In the *x*:*y* (Δ*z*-*cis*/*trans*) notation, *x* is the FA chain length, *y* is the number of double bonds, *z* is the position of the double bond relative to the carboxyl terminus, and *cis* or *trans* denotes the isomer of the double bond. In cases where only one name is given, both FA side chains of PC are the same. For POPC/SOPC, the FA chains are given in the order of carbons of the glycerol backbone (where the phosphocholine headgroup is bonded to the third carbon).

Shifting from a *Cis* to a *Trans* Isomer of a FA Chain Increases γ-Secretase Activity and Can Increase Aβ Processivity. To address this issue, we chose to study 16- and 18-carbon FA chains, as these are the most common lengths found in natural lipid bilayers. The mean γ-activity observed in the presence of each FA chain type, as determined by in vitro processing of recombinant APP substrate C100-FLAG to AICD-FLAG and Aβ, was compared to the mean activity of a simultaneously analyzed standard lipid condition, namely 100% POPC {palmitic/oleic acid [16:0/18:1 (Δ9-*cis*)] phosphatidylcholine} or 100% SOPC {stearic/oleic acid [18:0/18:1 (Δ9-*cis*)] phosphatidylcholine}.

Phospholipid mixtures of 90% POPC and 10% of one of four different FA isomers we tested [PC 16:1 (Δ9-*cis*), 16:1 (Δ9-*trans*), 18:1 (Δ9-*cis*), or 18:1 (Δ9-*trans*)] had little effect on AICD or Aβ production versus the 100% POPC standard (Figure 2A–C, red bars). Increasing the proportion of three of these four test isomers to 20% and then 50% led to progressive declines in AICD and $Aβ_{40}$ production, while $Aβ_{42}$ production was unchanged (Figure 2A,B, blue and green bars). The one exception to these declines occurred with PC 18:1 (Δ9-*trans*), mixtures of which actually increased the level of $Aβ_{40}$ relative to the control (90:10, $p < 0.05$; 80:20, $p < 0.001$) and to PC 18:1 (Δ9-*cis*) ($p < 0.001$). However, there were no significant increases in AICD and $Aβ_{42}$ levels (Figure 2A–C, right-most sets of bars). When we increased the concentration of each of the four test isomers to 100% (yellow bars), the first three isomers showed steep declines in the levels of AICD and $Aβ_{40}$ and an ∼50–100% decrease in the level of $Aβ_{42}$ ($p < 0.001$). However, the PC 18:1 (Δ9-*trans*) isomer was again exceptional: at a concentration of 100%, it actually increased $Aβ_{40}$ production by 50% ($p < 0.05$) and left $Aβ_{42}$ production unchanged. In the absence of POPC, shifting from a *cis* to a *trans* isomer in both PC 16:1 (Δ9) and PC 18:1 (Δ9) led to a significant increase in the level of generation of $Aβ_{42}$ ($p < 0.001$) (Figure 2C, yellow bars). Generally similar results were obtained when SOPC rather than POPC served as the standard phospholipid. The same relative changes in $Aβ_{40}$ and $Aβ_{42}$ levels were seen in an SOPC background, with the exception that at all concentrations tested, PC 18:1 (Δ9-*trans*) exhibited no difference in $Aβ_{40}$ production versus the 100% SOPC control (data not shown). Taken together, these initial results indicate that the isomer of a double bond in a phospholipid FA chain can directly and significantly influence γ-secretase activity.

We used the Aβ ELISA data given above to calculate $Aβ_{42}$:$Aβ_{40}$ ratios, elevations of which are observed in AD patients with inherited presenilin mutations. Increasing concentrations of PC 16:1 (Δ9-*cis*) and PC 16:1 (Δ9-*trans*) increased the $Aβ_{42}$:$Aβ_{40}$ ratio over that of the 100% POPC control, so that 50:50 mixtures of either of these with POPC increased the ratio by ∼2–3-fold (Figure 2D, green bars). In the case of PC 18:1 (Δ9-*trans*), its enhancement of γ-cleavage (levels of both AICD and $Aβ_{40}$ increased) led to an ∼20% decrease in the $Aβ_{42}$:$Aβ_{40}$ ratio. When SOPC rather than POPC was the standard lipid (Figure 2E), an elevated $Aβ_{42}$:$Aβ_{40}$ ratio was observed at 50% PC 16:1 (Δ9-*cis*) but not PC 16:1 (Δ9-*trans*), and PC 18:1 (Δ9-*trans*) again caused a small decrease. At 100% PC 16:1 (Δ9-*trans*), levels of $Aβ_{40}$ and $Aβ_{42}$ were very low, but the $Aβ_{42}$:$Aβ_{40}$ ratio was 6-fold ($p < 0.001$) and 3.5-fold ($p < 0.001$) higher than the 100% POPC and SOPC standards, respectively (Figure 2D,E, yellow bars); that is, although the

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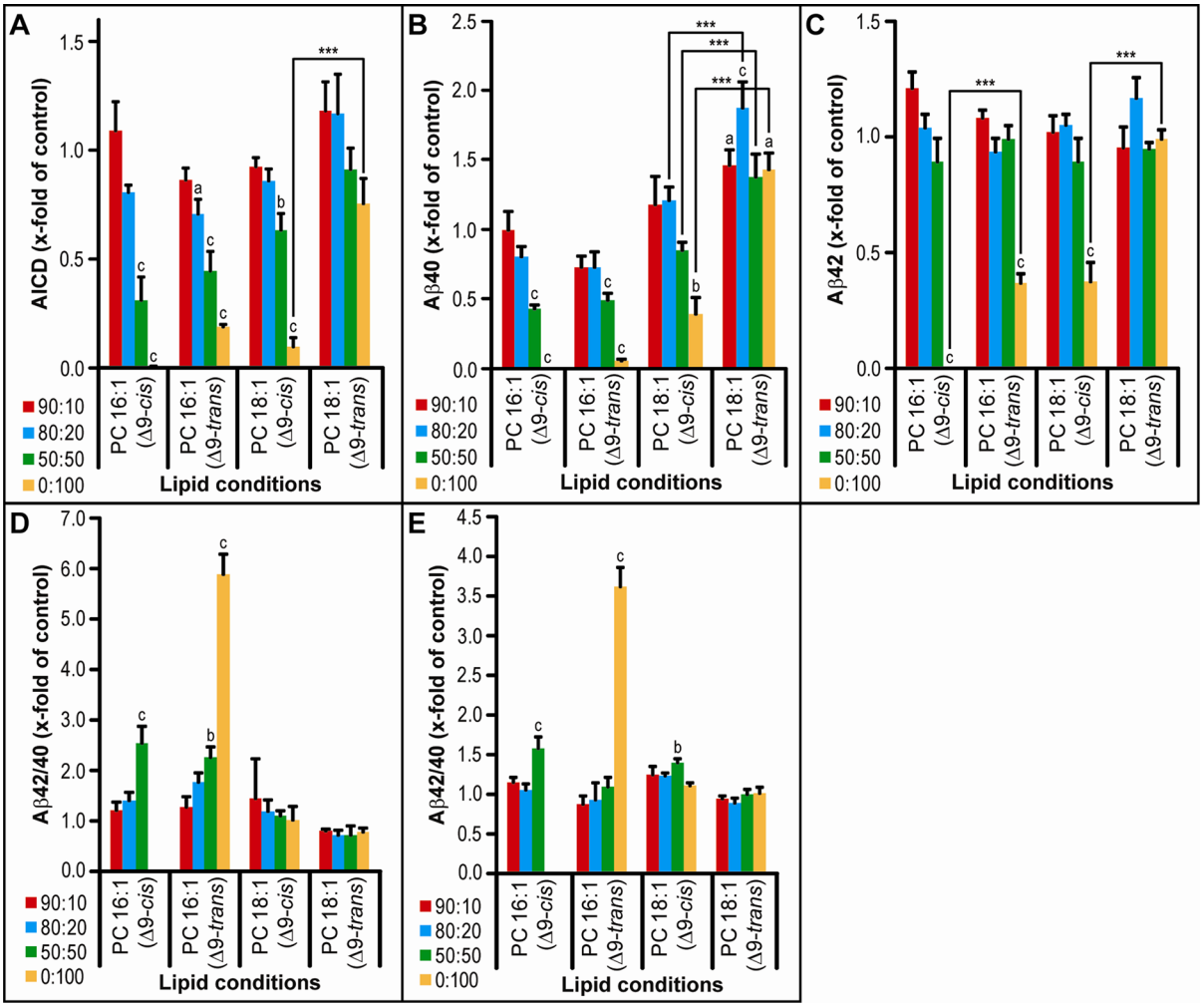

Figure 2. Effects of *cis* vs *trans* isomers of FA side chains on γ-secretase activity. Activity assays using purified γ-secretase and the C100-FLAG substrate were performed in detergent-free vesicles comprising different ratios of various FA-containing PCs at a total lipid concentration of 1.5 mM. Assay solutions were analyzed by (A) AICD-FLAG Western blot densitometry as a measure of total γ-activity, (B) an Aβ_{40} ELISA, and (C) an Aβ_{42} ELISA, allowing the calculation of Aβ_{42}:Aβ_{40} ratios for (D) the POPC background and (E) the SOPC background. Ratios given in the key denote the percentage of POPC (first number) and specific FA being tested (second number). Error bars give standard errors. Data were analyzed by one-way ANOVA with Bonferroni post-test correction. a−c (compared to the 100% control lipid) and one, two, and three asterisks (compared between lipids) denote $p < 0.05$, $p < 0.01$, and $p < 0.001$ statistical significance, respectively (n = 3−12).

overall level of Aβ production was reduced, the Aβ_{42}:Aβ_{40} ratio was shifted toward a more pathogenic one. Another interesting finding ensued when the position of the double bond in PC 18:1 (Δ9-*cis*) was shifted further from the center of the membrane bilayer (to the sixth carbon atom from the carboxyl group). In this PC 18:1 (Δ6-*cis*) environment, there was very limited γ-secretase activity either at 100% or with 50:50 mixtures with POPC (Figure 1 of the Supporting Information), demonstrating that the position of the double bond-induced kink can influence activity. This could be due to a decrease in membrane fluidity, as predicted because the longer stretch of acyl chain is uninterrupted by a double bond.[39] When compared with PC 18:1 (Δ9-*cis*), the Δ6-*cis* isomer led to significant reductions at an 80:20 ratio in the level of Aβ_{40} ($p <$ 0.001), at a 50:50 ratio in the level of AICD, Aβ_{40}, and Aβ_{42} (p < 0.001), and at a 0:100 ratio in the level of Aβ_{40} ($p < 0.01$) and Aβ_{42} ($p < 0.001$) (Figure 1 of the Supporting Information).

There is growing evidence that quantifying only Aβ_{40} and Aβ_{42} oversimplifies Aβ production,[13] as exemplified by recent reports regarding the potential pathogenicity of Aβ_{43}.[12] To examine the production of Aβ species of various C-terminal lengths, we employed a bicine/urea PAGE system.[13,40] A representative gel (Figure 3A) shows that a 50:50 mixture of PC 18:1 (Δ9-*cis*) with POPC decreased the level of Aβ_{40}, while longer species, including Aβ_{42} and Aβ_{43}, do not appear to be changed, in agreement with our ELISA data (Figure 2B,C). PC 18:1 (Δ9-*trans*) induced no change in Aβ_{42} but a slight increase in the level of Aβ_{40} and a corresponding decrease in the level of Aβ_{43} (Figure 3A). Using many such gels, we performed densitometry and estimated the relative levels of three groups of Aβ peptides: Aβ_{40}, Aβ_{42} and Aβ_{43}, and Aβ_{45} and longer forms (Figure 3B,C). This semiquantitative method revealed a relative decrease in the production of longer Aβ species (both 42/43 and 45+) and a relative increase in the production of Aβ_{40} as the percentage of PC 18:1 (Δ9-*trans*) increased, compared to the effects of POPC/PC 18:1 (Δ9-*cis*) mixtures or the standard POPC alone (Figure 3B,C). This effect of the *trans* isomer of PC 18:1 (Δ9) should be antiamyloidogenic, because the relative levels of longer, more hydrophobic Aβ species are diminished. In the case of PC 16:1 (Δ9), the ratios of longer

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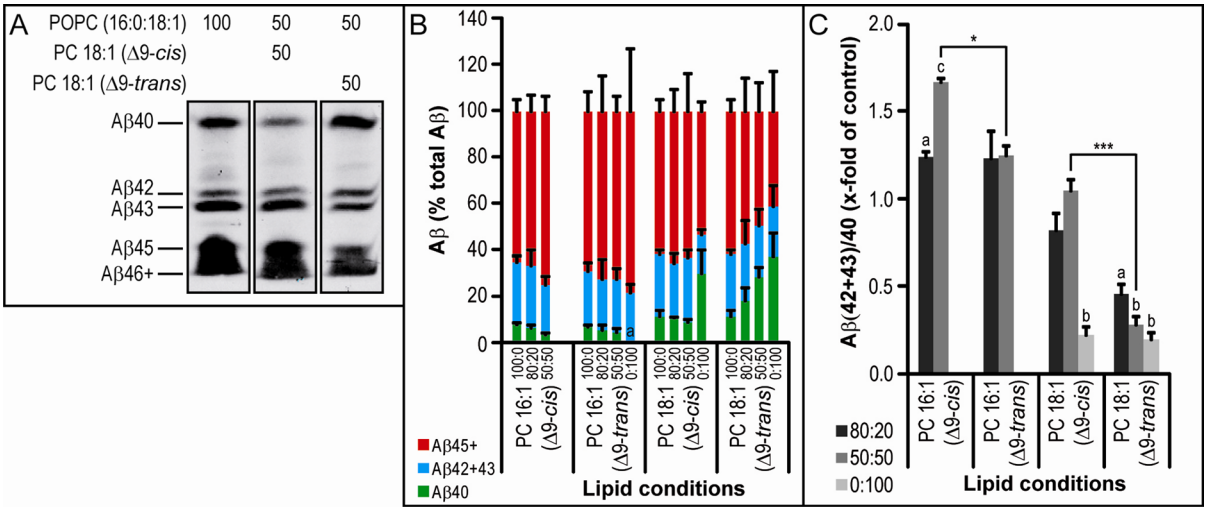

Figure 3. Effect of *cis* vs *trans* isomer FA side chains on γ-secretase processivity. Activity assay samples were run on bicine/urea gels, transferred to PVDF, and then probed for human Aβ (using the 6E10 antibody). (A) Part of one blot is shown as an example. (B) All blots were analyzed by densitometry and the results plotted as a percentage of the sum of all Aβ bands in a lane in groups of Aβ_{40} (green bars), Aβ_{42} and Aβ_{43} (blue bars), and Aβ_{45} and Aβ_{46+} (red bars). (C) A$\beta_{42/43}$:Aβ_{40} ratios were calculated from the densitometry data. Ratios below denote the percentage of POPC and specific FA chain being tested. In cases where no bar is shown, the band intensity was too low for detection by Western blot. Statistical analysis was performed as described in the legend of Figure 2 (n = 3).

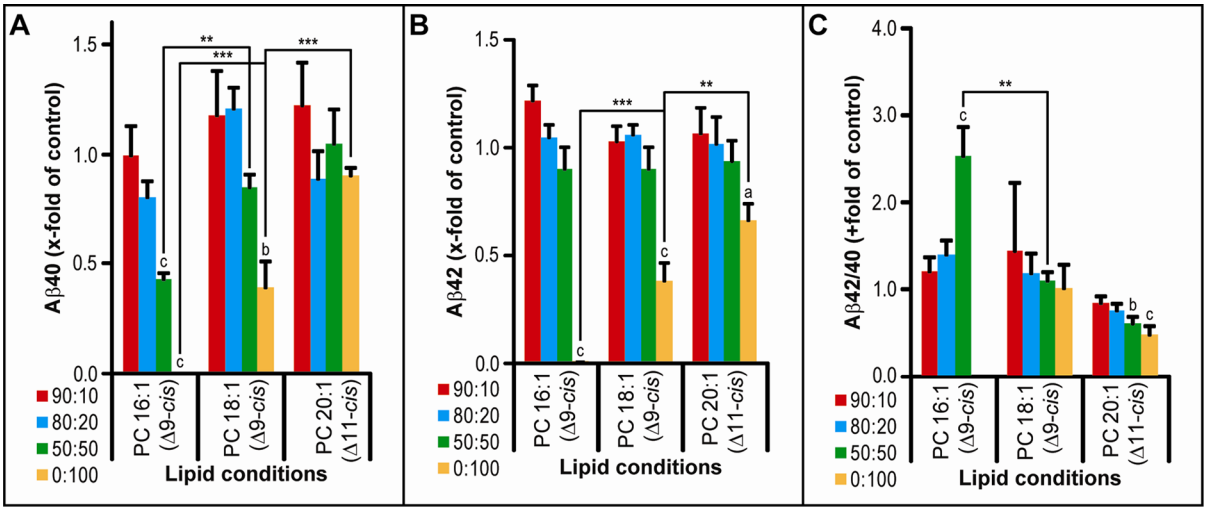

Figure 4. Effect of FA chain length on γ-secretase activity. Activity assays were analyzed by the same method as in Figure 2B–D.

Aβ peptides to Aβ_{40} were high (>1.0) with both the *cis* and *trans* isomers, suggesting a decreased processivity of γ-secretase in the presence of this phospholipid (Figure 3C). Unlike with the ELISA-calculated ratios, these data reveal a difference between the *cis* and *trans* isomers in 50:50 mixtures with POPC. Under these conditions, the *trans* form of both 16:1 (Δ9) and 18:1 (Δ9) results in a significant reduction in the A$\beta_{42/43}$:Aβ_{40} ratio relative to the effect of the *cis* isoform (p < 0.05 and p < 0.001, respectively) (Figure 3C). Thus, the orientation of a FA chain double bond as well as chain length can substantially alter the relative production of longer versus shorter Aβ peptides by purified γ-secretase.

An Increasing FA Chain Length Enhances γ-Secretase Activity and Processivity and Lowers the Aβ_{42}:Aβ_{40} Ratios. In addition to FA isomer differences altering membrane density and fluidity, FA chain lengths have effects on fluidity as well as membrane thickness. The latter parameter would be expected to have a profound effect on an intramembrane protease that cleaves sequentially at different points along the substrate's membrane-spanning domain. In the previous section, we saw that in a membrane of 100% PC 16:1 (Δ9-*cis*), there was no detectable γ-secretase activity, and a 50:50 mixture with POPC still left activity at less than half of that in pure POPC (Figure 2A). When we examined the effects of the Δ9-*cis* FA chain being extended serially by two carbons (i.e., 16 vs 18 vs 20 vs 22), we observed a stepwise increase in the production of Aβ_{40} (p < 0.001), even when PC 20:1 (Δ11-*cis*) phospholipids constituted 100% of the membrane (Figure 4A). Under the latter condition, the sole availability of the Δ11-*cis* form of PC 20:1 may mean that some of the observed effect is due to the change in the position of the double bond. When 22 carbons were used, there was a 40% decrease in activity (as measured by AICD-FLAG Western blot) with 50:50 mixtures containing PC 22:1 (Δ13-*cis*) relative to those containing PC 20:1 (Δ11-*cis*) (p < 0.001), probably because of the high phase transition temperature of PC 22:1 (data not shown). At just 14

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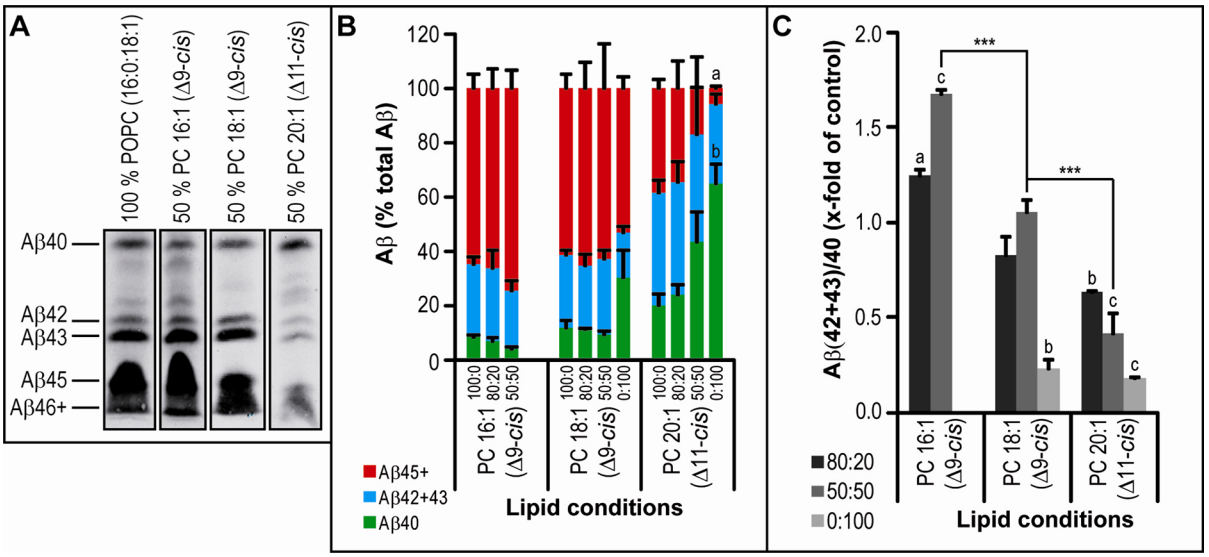

Figure 5. Effect of FA chain length on γ-secretase processivity. Activity assays were analyzed by the same method as in Figure 3A–C.

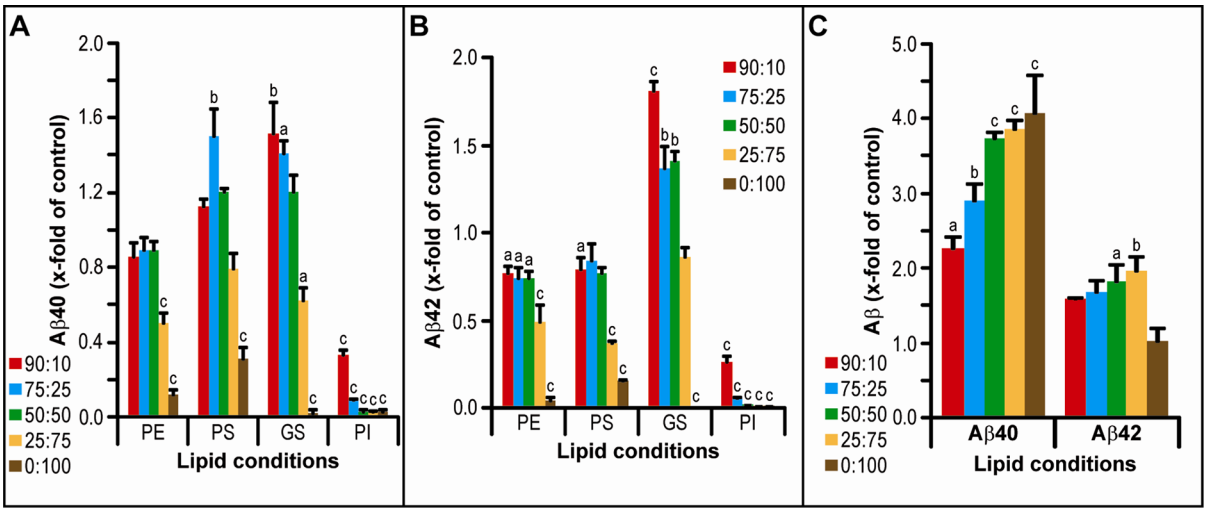

Figure 6. Effect of various lipid headgroups on γ-secretase activity. Activity assays using purified γ-secretase and C100-FLAG substrate were performed in detergent-free vesicles comprising different ratios of various lipid types (A and B) mixed with whole brain PC extract at a total lipid concentration of 1.5 mM. Activity assay samples were analyzed by the same method as in Figure 2B,C. (C) $A\beta_{40}$ and $A\beta_{42}$ ELISA data for whole brain lipid extract in a separate graph because of the different *y*-axis scale required. Statistical analyses were performed as in Figure 2 (n = 3–6).

carbons, production fell to two-thirds of control levels in 80:20 mixtures containing PC 14:1 (Δ9-*cis*) and almost to zero in 50:50 mixtures ($p < 0.001$), with a significant reduction in levels of $A\beta_{40}$ ($p < 0.01$) and $A\beta_{42}$ ($p < 0.001$) and an increase in the $A\beta_{42}$:$A\beta_{40}$ ratio ($p < 0.001$) relative to PC 16:1 (Δ9-*cis*) (Figure 2 of the Supporting Information). Production of $A\beta_{42}$ was essentially unchanged in mixtures containing FA chains of increasing length, but at 100% of the test FA chain, $A\beta_{42}$ levels did increase [$p < 0.001$ for PC 18:1 (Δ9-*cis*) vs PC 16:1 (Δ9-*cis*), and $p < 0.01$ for PC 20:1 (Δ11-*cis*) vs PC 18:1 (Δ9-*cis*)] (Figure 4B, yellow bars). With a smaller increase in the level of $A\beta_{42}$ relative to the level of $A\beta_{40}$, the $A\beta_{42}$:$A\beta_{40}$ ratio decreased to half the control level in the presence of the longer 20:1 (Δ11-*cis*) ($p < 0.001$) (Figure 4C). Conversely, reducing the the FA chain length from 18 to 16 carbons led to a 2.5-fold increase in the $A\beta_{42}$:$A\beta_{40}$ ratio ($p < 0.01$), primarily because of a decrease in $A\beta_{40}$ production with no change in $A\beta_{42}$ levels (Figure 4A–C). Further reducing the FA chain length to 14:1 (Δ9-*cis*) increased the $A\beta_{42}$:$A\beta_{40}$ even more to ~4.5 times the control levels in 50:50 mixtures ($p < 0.001$) (Figure 2 of the Supporting Information).

Using the bicine/urea gel system to examine longer Aβ peptides, we found that a membrane composed of 50% PC 20:1 (Δ11-*cis*) and 50% POPC led to a striking reduction in the levels of longer Aβ species ($A\beta_{42+}$) with no change in the levels of $A\beta_{40}$ (Figure 5A,B). In accord with the ELISA data, the $A\beta_{42/43}$:$A\beta_{40}$ ratio fell stepwise with an increasing chain length ($p < 0.001$), reaching low (physiological) levels in a membrane of 100% PC 20:1 (Δ11-*cis*) (Figure 5B).

Variations in Phospholipid Headgroups Alter γ-Secretase Activity. Another important feature of membrane lipids is the identity of polar headgroups, which can vary from the simple choline in phosphatidylcholine (PC) to a complex oligosaccharide in gangliosides (GS). These lipid headgroups are known to play roles in the interaction of proteins with the membrane, but how they affect intramembrane proteolysis by γ-secretase is not well understood. Previous work by our group[33] showed that mixtures of different lipid headgroup types

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can significantly alter the degree of proteolysis of C100 to AICD and Aβ_{40} by γ-secretase, but we did not examine relative changes among different Aβ peptide species. We have therefore revisited some of the conditions explored earlier by quantifying AICD-FLAG via Western blotting, Aβ_{40} and Aβ_{42} via an ELISA, and longer Aβ species via bicine/urea gels, all by supplementing whole brain PC with each of the test lipids described.

Densitometry of AICD-FLAG production from C100-FLAG by γ-secretase in various differently composed membranes revealed that only in a 90% PC/10% GS lipid mixture was an increase in total γ-activity observed ($p < 0.05$) (Figure 3A of the Supporting Information). At other PC:GS ratios and with all other lipid types tested [phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphoinositol (PI), phosphatidic acid (PA), sphingomyelin (SM), and cerebrosides (CS)], γ-activity was unaffected until the test lipid comprised more than 50% of the membrane, at which point a marked reduction in the levels of all γ-products was observed (Figure 3A of the Supporting Information). As was previously reported by Osawa et al.,[36] phosphatidylinositol (PI) markedly inhibited γ-activity, with as little as 10% in the membrane causing a complete loss of AICD generation (Figure 3A of the Supporting Information). We found a similar but less potent negative effect from phosphatidic acid (PA): γ-activity was completely abrogated at a 50% membrane composition (Figure 3A of the Supporting Information). Analyzing the same samples with an Aβ ELISA revealed similar patterns of effects, except that in the cases of phosphatidylserine (PS) and PA, we saw an increase in Aβ_{40} production (vs control; $p < 0.01$) with a 75:25 PC:PS or a 75:25 PC:PA ratio (Figure 6A and Figure 3B of the Supporting Information). Interestingly, GS was the only lipid type to increase Aβ_{42} production over a wide range of lipid ratios, while all other lipid headgroups (PE, PS, SM, PI, and PA) decreased it (Figure 6B and Figure 3C of the Supporting Information).

To examine a potentially more physiological lipid condition for γ-secretase activity, we also performed in vitro activity assays with increasing percentages (again within a PC membrane) of whole porcine brain (WB) lipid extract, which is a complex mixture of lipids containing all of the lipids we have tested individually above. By both AICD and Aβ_{40} measures, we found a stepwise increase in γ-activity with each increase in percentage of WB present in the PC membrane, peaking at a value 4 times the control levels (Figure 6C; AICD data not shown). An Aβ_{42} ELISA revealed a smaller increase (to ~2-fold) for 25:75 PC/WB lipid mixtures, whereas going to 100% WB lipids decreased Aβ_{42} production to control (i.e., 100% PC) levels (Figure 6C).

Calculating Aβ_{42}:Aβ_{40} ratios based on all of the ELISA data shown in Figure 6 revealed that PE and SM did not change this ratio from controls, while GS increased it as much as 2-fold (when GS was 75% of the mixture with PC; $p < 0.001$) (Figure 7B and Figure 4 of the Supporting Information). With increasing amounts of whole brain lipids, we observed significant stepwise decreases in Aβ_{42}:Aβ_{40} ratios to a final level that was ~20% of the PC control at 100% WB lipid ($p < 0.001$). This pattern was similar with PS, PI, and PA, although the latter two headgroups gave low levels of total activity (Figure 7B and Figure 4 of the Supporting Information). Samples were further analyzed on bicine/urea gels (Figure 7A,D), which likewise showed that PE and SM did not significantly alter the A$\beta_{42/43}$:Aβ_{40}, and both PS and WB significantly ($p < 0.001$) reduced the ratio (Figure 7C). Unexpectedly, the densitometry data from these blots did not agree with the ELISA data, largely because of a high level of

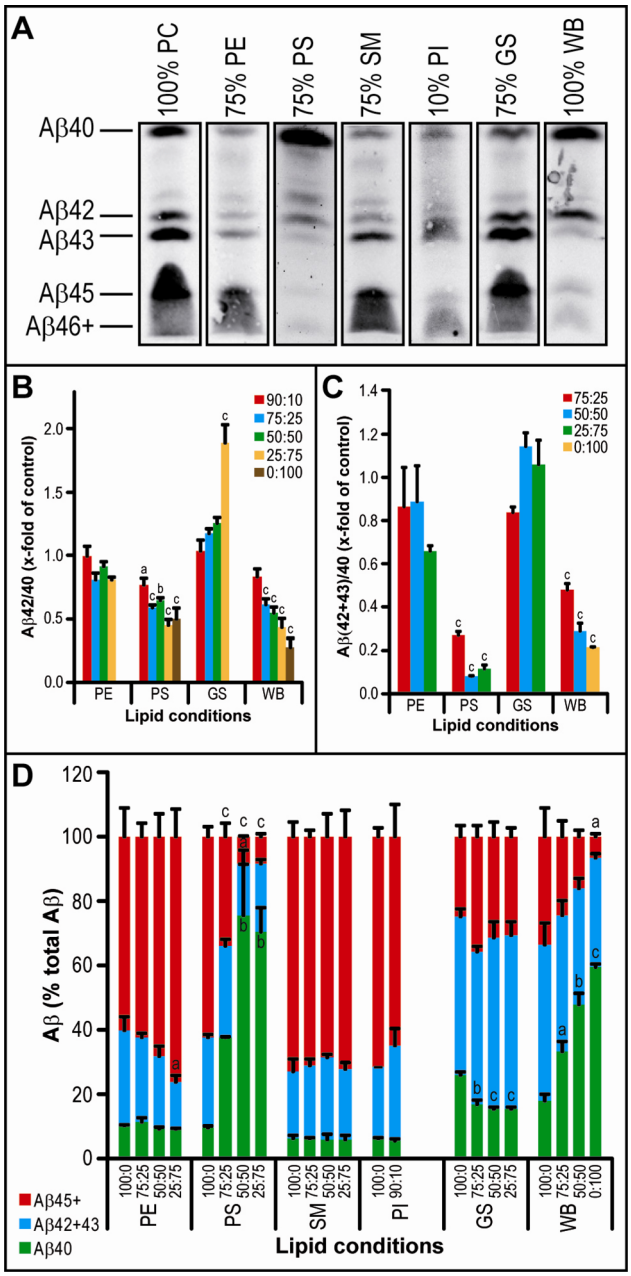

Figure 7. Effect of lipid headgroups on γ-secretase processivity. Activity assay samples were analyzed by the same method as in Figures 2D and 3A–C.

Aβ_{43} production (a species that is not detected by the ELISA) in both 75% GS and control membranes (Figure 7D). The WB lipid condition gave a pattern of Aβ species much closer to the physiological pattern, with an ~10% Aβ_{42}:Aβ_{40} ratio and trace levels of the longer Aβ species (Figure 7A,C,D). The only individual lipid headgroup giving a pattern similar to that of WB lipids was a high level of PS (Figure 7A,C,D), suggesting that PS could be an important lipid type for correct γ-secretase processing of the substrate, although the levels of Aβ production in pure PS were greatly reduced compared to that of WB and the 100% PC control.

Exposing Living Cells to Polyunsaturated Fatty Acyl Side Chains Decreases Aβ Production and Increases the Aβ_{42}:Aβ_{40} Ratio. The experiments described above sought to determine the effects of lipid variation directly on the activity of

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purified γ-secretase. We also examined the effect of certain fatty acyl side chains on the γ-secretase processing of APP in the context of living cells. Chinese hamster ovary (CHO) cells stably overexpressing all four components of human γ-secretase as well as human APP[34] were treated for 48 h with different phospholipids (final concentration of 10 or 50 μM) in vesicles prepared by hydration of the dried lipid and sonication, or else just with vehicle (the buffer used for hydrating the lipids). The resultant conditioned media (CM) were quantified for Aβ$_{40}$ and Aβ$_{42}$. We found that treatment of the cells with PC 16:1 (Δ9-*cis*) or (Δ9-*trans*) in vesicles had no major effect on Aβ$_{40}$ and Aβ$_{42}$ levels compared to vehicle alone (Figure 8A) but did

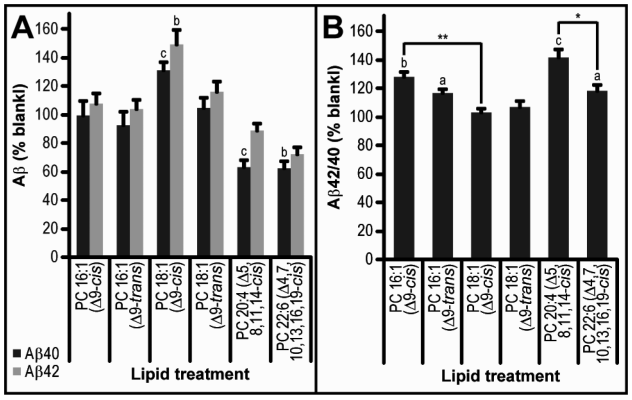

Figure 8. Effect of FA chain length and saturation on γ-secretase activity in living cells. S20 CHO cells were treated with 50 μM lipid vesicles in standard growth medium for 48 h. Conditioned media were analyzed by a 4G8 triplex Aβ ELISA (Mesoscale Discovery) and values normalized to the BCA assays of the cell lysates. (A) Aβ levels are given as a percentage of normalized Aβ readings from vehicle-treated controls from the same plate. (B) Aβ$_{42}$:Aβ$_{40}$ ratio calculated from non-normalized readings and then given as a percentage of vehicle-treated controls from the same plate. Statistical analysis was performed as in Figure 2 (n = 5−12).

slightly increase the Aβ$_{42}$:Aβ$_{40}$ ratio by 30% (p < 0.001) or 15% (p < 0.05), respectively (Figure 8B). However, treatment with the longer PC 18:1 (Δ9-*cis*) elevated both Aβ$_{40}$ and Aβ$_{42}$ levels by 30−40% (for Aβ$_{40}$, p < 0.001; for Aβ$_{42}$, p < 0.01), while its *trans* isomer produced no change from control levels (Figure 8A), with neither isomere significantly altering Aβ$_{42}$:Aβ$_{40}$ ratios relative to the control (Figure 8B). Interestingly, the earlier in vitro observation that a decreasing FA chain length increases the Aβ$_{42}$:Aβ$_{40}$ ratio was also found upon comparison of intact cells treated with PC 18:1 (Δ9-*cis*) versus PC 16:1 (Δ9-*cis*) (p < 0.01) (Figure 8B). Exposing the cells to the polyunsaturated fatty acyl side chain arachidonic acid (AA) [i.e., PC 20:4 (Δ5,8,11,14-*cis*)] at 50 μM (but not 10 μM) reduced the level of Aβ$_{40}$ by 30% (p < 0.001) while not significantly changing the level of Aβ$_{42}$ (Figure 8A). Treatment with polyunsaturated FA side chain docosahexaenoic acid (DHA) [i.e., PC 22:6 (Δ4,7,10,13,16,19-*cis*)] at 50 μM (but not 10 μM) decreased the levels of both Aβ$_{40}$ and Aβ$_{42}$. In accord with these data, the Aβ$_{42}$:Aβ$_{40}$ ratio was elevated by ∼40% with AA (p < 0.001) but only slightly elevated with DHA (p < 0.05), showing a significant difference between these two PUFA exposures (p < 0.05) (Figure 8B).

DISCUSSION

Using a modification of a detergent-free γ-activity assay devised in our lab,[33] we find that the lipid microenvironment surrounding γ-secretase has a very important and complicated role in the activity of this intramembrane protease. This study shows that both the fatty acyl chains and the polar headgroups of lipids can critically regulate the proteolysis. There have been relatively few studies on the in vivo effects of FA chains (with DHA being the most studied) or lipid headgroups in animals and humans, largely because of the longitudinal nature of such studies and the difficulty of fully controlling dietary lipid intake. In this study, we applied a reductionist approach by studying purified γ-secretase activity in a controlled system with specific pure lipids and purified enzyme. This approach avoids many of the confounding effects found in vivo such as altered protein expression and trafficking, interaction with other proteins, and cell toxicity. Most in vitro studies of γ-secretase activity have used proteins in the partially purified state in isolated cellular membranes, which contain many other membrane-bound proteins and an abundance of lipids, all of which make it difficult to discern any effects of specific lipids on protease activity. Furthermore, without removing the invariably used detergents from the system, the assay actually measures the effect of lipid/detergent mixtures on γ-secretase activity and not solely the effects of the lipids.

As membrane lipids consist of a polar headgroup and a hydrophobic tail, we asked whether one or both of these constituents could play a role in the activity of an intramembrane protease such as γ-secretase. An example of this approach was reported by Contreras et al., who found a transmembrane domain sequence of p24 [involved in coat protein (COPI) vesicle biogenesis] to which the polar headgroup and FA chain of SM-18:5 directly and specifically bind, leading to efficient retrograde COPI-dependent transport.[41] To similarly investigate the role of membrane lipids in γ-secretase function, we separated our experiments into two parts, first systematically varying the FA chain isomerization and length with a constant headgroup (PC) and then examining various different lipid headgroups.

We found that changing a monounsaturated FA isomer from *cis* to *trans* with a constant chain length led to an increase in the extent of γ-secretase cleavage of the C100-FLAG substrate, measured by AICD Western blotting and Aβ$_{40}$ and Aβ$_{42}$ ELISAs, without significantly affecting the Aβ$_{42}$:Aβ$_{40}$ ratio. However, upon closer examination using a bicine/urea gel system in which a range of Aβ peptide lengths can be resolved, we observed that shifting from *cis* to *trans* FA isomer reduces Aβ$_{43}$ production and, in the case of PC 18:1 (Δ9), also reduces the level of Aβ species with ≥45 residues. Following the Ihara model of sequential, N-terminally directed cleavage of the APP TMD starting at Aβ$_{48}$ or Aβ$_{49}$,[40] our results can be explained as the *trans* isomer increasing processivity, that is, increasing the extents of the Aβ$_{45/48}$ to Aβ$_{42}$ and Aβ$_{43/46/49}$ to Aβ$_{40}$ conversions. An alternative explanation not implicating a processive cleavage mechanism could be one in which alterations in the lipid microenvironment affect the precise site of γ-cleavage through local changes in membrane thickness, leading to differential alignments of the catalytic aspartate residues with the substrate's transmembrane domain. However, given the clear evidence of release of tri- and tetrapeptides from the C99 transmembrane domain during γ-secretase cleavage[11]

and the changes in Aβ species seen on our bicine/urea gels, we favor the model of sequential processing of the substrate.

When analyzing our reconstituted γ-secretase system for effects of FA chain length, we observed a bell shape of γ-activity peaking at lengths of 18 or 20 carbons, with very low activity at 14 carbons and decreased activity at 22 carbons. The most common length for FA chains in humans is in the range of 16−18 carbons. We also observed a decrease in the $A\beta_{42}$:$A\beta_{40}$ ratio (measured by an ELISA) and a reduced $A\beta_{42/43}$:$A\beta_{40}$ ratio and loss of $A\beta_{45+}$ peptides (measured by bicine/urea PAGE) as the FA chain length increased, suggesting longer FA chains allow more physiological processing of the C99 APP substrate. The discrepancy between this higher processivity to shorter Aβ peptides and natural FA chain length patterns in cells may be due to the localization of γ-secretase and the substrate to detergent-resistant membranes (DRMs), which are less fluid and contain higher levels of the longer 20- and 22-carbon FA chains.[42] A mechanistic explanation of our data could be that with a thicker hydrophobic region and the reduced fluidity of a membrane, the longer Aβ species ($A\beta_{45+}$) may be better retained in the γ-secretase complex and therefore undergo subsequent γ-cleavage to the shorter (and less amyloidogenic) species, $A\beta_{40}$ and $A\beta_{38}$. In terms of establishing a general model of how the lipid microenvironment affects γ-secretase function, it will be necessary to perform these assays with additional substrates, such as Notch, which is implicated in several types of cancer. Our study has focused on the role of γ-secretase relevant to Alzheimer's disease, but we assume that the processivity model may turn out to be universal for most if not all substrates, based on the observations of Fukumori et al.[43] that even the autoproteolysis of PS1 occurs in a stepwise manner with cleavages every three residues.

Another principal thrust of our study was to expand on previous work[33] that examined the effects of various polar headgroups of membrane lipids on γ-secretase activity. Our previous work largely focused on the effect of cholesterol in conjunction with various different lipid types, showing that cholesterol causes a very significant increase (and then a decrease at higher concentrations in a bell-shaped response) in γ-secretase activity. The data shown here look at cholesterol-free proteoliposomes to investigate how lipid headgroups per se can influence γ-secretase activity and processivity. We have shown that when combined with PC, increasing proportions of PS, GS, or PA elevate $A\beta_{40}$ production in a bell-shaped fashion, while only GS increased $A\beta_{42}$ production. Correspondingly, the pathogenically important $A\beta_{42}$:$A\beta_{40}$ ratio was increased by high GS concentrations and decreased by PS and PA. These findings about the direct effects of GS on γ-secretase activity support studies in mice in which various enzymes of the ganglioside synthesis pathways have been knocked out. In particular, in vivo prevention of the generation of disialogangliosides (e.g., GD3, GD2, and GD1b), thereby elevating GM3 concentrations, can slow Aβ production and aggregation in APP/PS1 transgenic mice.[44,45] It may now be useful to investigate γ-secretase activity and processivity in proteoliposomes comprising specific ganglioside subtypes to reveal potential differences.

Intriguingly, PS was the only lipid headgroup tested here that reduced relative levels of the very long Aβ species, $A\beta_{45+}$, to the same low levels as did a whole brain lipid extract, which mimics the physiological lipid environment of γ-secretase. This finding may be due to the tendency of PS to move to the inner leaflet of membranes, providing asymmetry to the bilayer,[46] and its anionic headgroup locking to C99 in the membrane more strongly because of the greater polarity between uncharged hydrophobic FA chains and the negatively charged headgroup, thus allowing further processing by γ-secretase. We also found that another anionic lipid type, PI, completely abrogates γ-secretase activity even at very low concentrations. This finding is in agreement with the work of Osawa et al.,[36] who discovered that phosphatidylinositol (3,4)/(4,5)-diphosphate (PIP_2) and PIP_3 directly inhibit γ-secretase activity in a competitive manner and that the inhibitory potency increases with the level of PIP phosphorylation and requires the FA side chains. Perhaps a reduction in PI levels, which has been reported in the anterior temporal cortex of AD brains,[47] would reduce the level of PI-mediated inhibition of γ-secretase, thus leading to an increase in Aβ production. Moreover, a relative loss of PS, as observed in AD mouse models,[48,49] may elevate the proportion of longer Aβ species, resulting in a more pathogenic Aβ profile. This hypothetical formulation mentions just two types of lipid headgroups. When the numerous lipid headgroup types (e.g., PC, PE, GS, SM, etc.), each containing various different subtypes and with two FA side chains that can vary in length, saturation, and isomerization, are taken into account, the complexity of the role of membrane lipids in γ-secretase activity and processivity becomes apparent. Recently, Nesic et al.[37] used siRNA-mediated knockdown of PE synthesis in cells and *Drosophila melanogaster* to reduce the rate of Aβ generation via an increased level of α-secretase cleavage and decreased γ-secretase processing of APP. This demonstrates that lipid modulation in vivo can have positive effects on Aβ production and potentially on AD pathogenesis.

In summary, we have shown that the lipid microenvironment in which γ-secretase is situated can have direct and potent effects on overall Aβ generation as well as processivity and the proportion of different length Aβ peptides; both factors are implicated in AD pathogenesis. The effects we document involve several aspects of membrane lipids, including the length of FA chains, their degree of unsaturation, their *cis* versus *trans* isomerization, and the identity of the polar headgroups. The central conclusion of our systematic analyses is that variation of membrane lipid composition could have effects on the pathogenic ratio of longer to shorter Aβ peptides that are equal to or more adverse than the effects of presenilin mutations or other protein changes that have been studied more extensively. Accordingly, our data recommend attempting specific manipulations of membrane lipid composition by using genetic and dietary approaches in animals. The findings also suggest specific targets for small molecule inhibitors to modify lipid biosynthesis pathways as well as lipid dietary changes that might similarly delay the onset or slow the progression of Alzheimer's disease.

■ ASSOCIATED CONTENT

Supporting Information

The effect of shifting the position of the double bond in PC 18:1 (Δ-*cis*) on γ-secretase activity (Figure 1), a comparison of the effect of PC 14:1 (Δ9-*cis*) versus PC 16:1 (Δ9-*cis*) on γ-secretase activity (Figure 2), the effect of lipid headgroups on γ-secretase activity (AICD production) and the effect of SM and PA on $A\beta_{40}$ and $A\beta_{42}$ generation (Figure 3), and the effect of SM, PI, and PA on γ-secretase processivity (Figure 4). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS

AD, Alzheimer's disease; FA, fatty acyl; APP, β-amyloid precursor protein; Aβ, amyloid β-peptide; AICD, APP intracellular domain; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; GS, gangliosides; PI, phosphatidylinositol; WB, whole brain lipid; SM, sphingomyelin; PA, phosphatidic acid; ELISA, enzyme-linked immunosorbent assay; CHAPSO, 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonic acid.

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