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Synthetic ceramide analogues increase amyloid-β 42 production by modulating γ-secretase activity

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ABSTRACT

γ-Secretase cleaves amyloid β-precursor protein (APP) to generate amyloid-β peptide (Aβ), which is a causative molecule of Alzheimer disease (AD). The C-terminal length of Aβ, which is determined by γ-secretase activity, determines the aggregation and deposition profiles of Aβ, thereby affecting the onset of AD. In this study, we found that the synthetic ceramide analogues DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) and (1*S*,2*R*-D-erythro-2-N-myristoylamino)-1-phenyl-1-propanol (DMAPP) modulated γ-secretase-mediated cleavage to increase Aβ42 production. Unexpectedly, PDMP and DMAPP upregulated Aβ42 production independent of alteration of ceramide metabolism. Our results propose that synthetic ceramide analogues function as novel γ-secretase modulators that increase Aβ42, and this finding might lead to the understanding of the effect of the lipid environment on γ-secretase activity.

1. Introduction

Amyloid-β peptide (Aβ) deposited in the brains of patients with Alzheimer disease (AD), is derived from amyloid β-precursor protein (APP) through sequential proteolytic cleavages by β- and γ-secretases [1]. β-site APP cleaving enzyme 1 (BACE1) is a type-1 transmembrane protein responsible for the β-secretase activity [2–4]. γ-Secretase is comprised of four integral membrane proteins, namely, Presenilin (the catalytic subunit), Nicastrin, Aph1, and Pen2 [5,6]. γ-Secretase cleaves a scissile bond within the transmembrane domain of APP and determines the C-terminal length of Aβ [1,7]. Mutations in Presenilin genes account for the majority of early onset familial AD (FAD) cases, by causing an overproduction of Aβ ending at position 42 (Aβ42) [8,9], which is the most amyloidogenic species of Aβ and is linked to the pathogenesis of AD [10].

The active site of Presenilin is embedded in the lipid bilayer. We and others showed that γ-secretase resides in lipid rafts [11,12], and this lipid raft localization of γ-secretase affects the metabolism of Aβ *in vitro* [13] and *in vivo* [14]. In particular, the components of sphingolipids, including ceramides that compose lipid rafts, were found to modulate γ-secretase activity in *in vitro* assays [13,15], suggesting that γ-secretase activity is modulated by the lipid microenvironment. In this study, we examined the effect on γ-secretase activity of DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) and (1*S*,2*R*-D-erythro-2-*N*-myristoylamino)-1-phenyl-1-propanol (DMAPP), which are synthetic ceramide analogues as well as inhibitors of glucosylceramide synthase (GCS) [16] and alkaline ceramidase (CDase) [17], respectively. These ceramide analogues increased the production of Aβ42 from cultured cells. Intriguingly, non-ceramide type inhibitors of GCS and alkaline CDase, and genetic suppression of GCS failed to upregulate Aβ42 production. We also found that these ceramide analogues promoted the Aβ42-generating γ-secretase activity in an *in vitro* assay. These data suggest that ceramide analogues directly modulate γ-secretase activity to increase Aβ42 production.

2. Material and methods

2.1. Compounds

PDMP (Enzo Life Sciences), (1*S*,2*R*-L-erythro-2-*N*-myristoylamino)-1-phenyl-1-propanol (LMAPP) (Enzo Life Sciences), *N*-buty-

Abbreviations: AD, Alzheimer disease; FAD, familial AD; Aβ, amyloid-β peptide; APP, amyloid precursor protein; BACE1, β-site APP cleaving enzyme 1; PDMP, DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; DMAPP, (1*S*,2*R*-D-erythro-2-*N*-myristoylamino)-1-phenyl-1-propanol; LMAPP, (1*S*,2*R*-L-erythro-2-N-myristoylamino)-1-phenyl-1-propanol; NB-DNJ, *N*-butyldeoxynojirimycin; NOE, *N*-oleoylethanolamine; GCS, glucosylceramide synthase; CDase, ceramidase; SC100, C-terminal 99 amino acid fragment of APP; HEK/NL, HEK293 cells stably expressing the Swedish mutant of APP.

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ldeoxynojirimycin (NB-DNJ; Sigma-Aldrich), *N*-oleoylethanolamine (NOE), DMAPP (Cayman Chemical), zVAD-FMK (MBL) were purchased from the indicated companies, and solubilized in DMSO. AlamarBlue (Life Technologies) was used as described previously [18].

2.2. Cell culture and transfection

Expression plasmids coding for human APP carrying the Swedish mutation (pcDNA3.1-APPNL) and carrying the APP C-terminal 99 amino acid fragment (pcDNA3.1-SC100) were described previously [18–20]. Plasmid transfection was performed using Lipofectamine2000 (Life Technologies). HEK293 cells and Neuro2a cells were maintained as described previously [21,22]. HEK293 cells stably expressing the Swedish mutant of APP (HEK/NL) was established by transfection of HEK293 cells with pcDNA3.1-APPNL followed by selection with G418. The glucosylceramide synthetase-deficient B16 melanoma mutant cell line GM95, and its parental cell line MEB4 [23] were supplied from Riken Bioresource Center Cell Bank (Tsukuba, Japan). GM95 and MEB4 were transiently transfected with pcDNA3.1-SC100 using Lipofectamine2000. Small interfering RNA (siRNA) duplexes targeting the human GCS sequence (target sequences: si-1: CAG GTG TCT CTC TTC TGA AAC CAC T; si-2: GCC AGG ATA TGA AGT TGC AAA GTA T; si-3: TGT GTG ACA GGA ATG TCT TGT TTA A), as well as a negative control sequence were purchased from Life Technologies. siRNAs were reversely transfected into HEK293 cells stably expressing the Swedish mutant of APP using Lipofectamine RNAiMax (Life Technologies) as previously described [20].

2.3. Immunological methods and cell-free analysis of γ-secretase activity

Samples were analyzed by two-site enzyme-linked immunosorbent assay (ELISA) or immunoblotting using the urea/SDS–PAGE gel system as described previously [21,22,24,25]. Aβ peptides and the APP intracellular domain (AICD) were detected by the anti-human Aβ antibody 82E1, and the APP C-terminus antibody APP(C), respectively (Immuno-Biological Laboratories). Horseradish peroxidase-conjugated cholera toxin B subunit (Sigma–Aldrich) was used for the detection of monosialotetrahexosylganglioside (GM1 ganglioside). Synthetic Aβ peptides were used as molecular standards. For the cell-free γ-secretase assay, membranes of Chinese hamster ovary (CHO) cells stably expressing C99 were collected and analyzed as described previously [22,26]. In all, microsomes (2.5 mg/ml) in homogenized buffer (20 mM HEPES (pH 7.0), 140 mM KCl, 250 mM sucrose, and 1 mM EGTA) containing 0.5 mM DIFP, 0.5 mM PMSF, 1 mg/ml TLCK, 1 mg/ml antipain, 1 mg/ml leupeptin, 10 mg/ml phosphoramidon, 1 mM EGTA, and 5 mM EDTA were pre-incubated with ceramide analogues on ice for 30 min, and then incubated at 37 °C for 6 h.

3. Results

3.1. Ceramide analogues PDMP and DMAPP increase the production of Aβ42

To examine the effect of ceramide metabolism on Aβ production, we analyzed the effect of the ceramide analogues PDMP and DMAPP, which are inhibitors of GCS [16] and alkaline CDase [17], respectively (Fig. 1A and B), on HEK293 cells stably expressing the Swedish mutant of APP (HEK/NL). As expected by the points of action of each reagent, we confirmed that the levels of GM1, an end product of glucosylceramide, showed a decrease and an increase by PDMP and DMAPP, respectively (Fig. 1C). We found that treatment with PDMP or DMAPP increased the secretion of Aβ, particularly the Aβ42 species, in a similar fashion to that by the known γ-secretase modulator fenofibrate (Fig. 1D and E). PDMP and DMAPP cause the accumulation of cellular ceramide [27], which stabilizes the BACE1 protein and increases Aβ production [28–30]. However, both PDMP and DMAPP dramatically increased Aβ42 production in HEK293 cells expressing the direct γ-secretase substrate SC100 (Fig. 1F and G). These results indicate that PDMP and DMAPP modulate γ-secretase activity to increase Aβ42 production.

3.2. Upregulation of Aβ42 production by ceramide analogues is independent of an apoptotic mechanism

APP overexpression affects cellular lipid metabolism [31]. Thus, we analyzed the effects of these compounds on mouse Neuro2a cells, which secrete endogenous Aβ. As in HEK293 cells, PDMP decreased, but DMAPP increased GM1 levels in Neuro2a cells (Fig. 2A). In addition, both ceramide analogues increased the ratio of Aβ42 production to total Aβ (Fig. 2B). The intracellular accumulation of ceramide by these ceramide analogues is known to induce apoptosis in neuronal cells [32,33]. However, neither PDMP nor DMAPP affected cell viability at the doses used (Fig. 2C). Furthermore, the pan-caspase inhibitor zVAD-FMK, which is known to inhibit apoptosis, did not alter the effect of DMAPP on Aβ production (Fig. 2D). These results suggest that upregulation of Aβ42 production by ceramide analogues is independent of the apoptotic process.

3.3. Increased Aβ42 production by ceramide analogues is independent of ceramide metabolisms

To analyze whether the accumulation of ceramide is associated with Aβ42 overproduction, we examined non-ceramide analogue inhibitors against enzymes involved in ceramide metabolism. The GCS inhibitor NBD-DNJ [34], and the acid ceramidase inhibitor *N*-oleoylethanolamine (NOE) [35] are known to increase ceramide levels. However, neither NBD-DNJ nor NOE altered Aβ production (Fig. 3A and B). Furthermore, the cell permeable C6 ceramide failed to affect Aβ production (Fig. 3A). Surprisingly, LMAPP, which is the stereo isomer of DMAPP and is inactive against alkaline CDase [17], was still capable of increasing Aβ42 production (Fig. 3D). These data strongly indicate that Aβ42 upregulation by ceramide analogues is dependent on their chemical structure, but not on aberrant ceramide metabolism.

3.4. PDMP and DMAPP directly modulate γ-secretase activity

To further confirm our hypothesis, we knocked down the expression of GCS in HEK/NL by siRNA transfection (Fig. 4A). Knockdown of GCS resulted in a decrease in GM1 levels in a similar manner to that by PDMP (Fig. 1C). However, no alteration in Aβ42 production ratio was observed. Next, we tested GM95 cells, which is a GCS-deficient B16 melanoma mutant cell line [23]. We transiently transfected GM95 and its parental cell line MEB4 with pcDNA3.1-SC100, but their ratios of Aβ42 to total Aβ was comparable (MEB4: 4.63 ± 0.59%; GM95: 4.67 ± 1.01%; Relative ratios are depicted in Fig. 4B).

To further examine the direct effect of ceramide analogues on γ-secretase activity, we performed a cell-free assay using the membrane fraction of CHO cells stably expressing SC100 [22]. We found that both PDMP and DMAPP increased Aβ42 production without affecting the production of the AICD (Fig. 4C). PDMP also increased the levels of Aβ39, which might be the byproduct of Aβ42 overproduction [13,25]. Taken together, our results suggest that ceramide analogues can modulate γ-secretase activity.

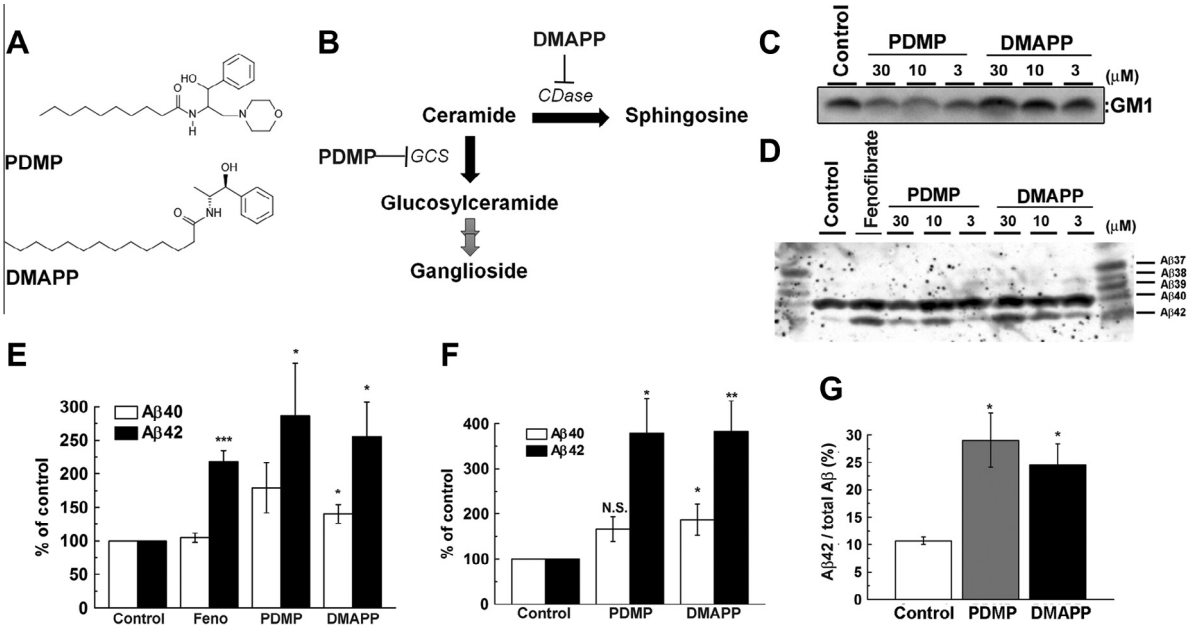

Fig. 1. Ceramide analogues PDMP and DMAPP increased Aβ42 by the modulation of γ-secretase activity. (A) Chemical structures of PDMP and DMAPP. (B) Schematic depiction of ceramide metabolism. PDMP and DMAPP inhibit GCS and alkaline CDase, respectively. (C–E) Effect of PDMP and DMAPP on HEK293 cells stably expressing the Swedish mutant of APP. (C) GM1 ganglioside levels in cell lysates were detected using the horseradish peroxidase conjugated cholera toxin subunit. (D) Immunoblot analysis of secreted Aβ in the culture medium. (E) Quantification of secreted Aβ by sandwich ELISA ($n = 4$, mean ± SEM; $^{*}P < 0.05$; $^{***}P < 0.001$). (F) Effect of PDMP and DMAPP on HEK293 cells transiently expressing SC100. Secreted Aβ was quantified by ELISA ($n = 4$, mean ± SEM; N.S.: not significant, $^{**}P < 0.01$). (G) Ratio of Aβ42 to total Aβ (Aβ40 + Aβ42) in (F).

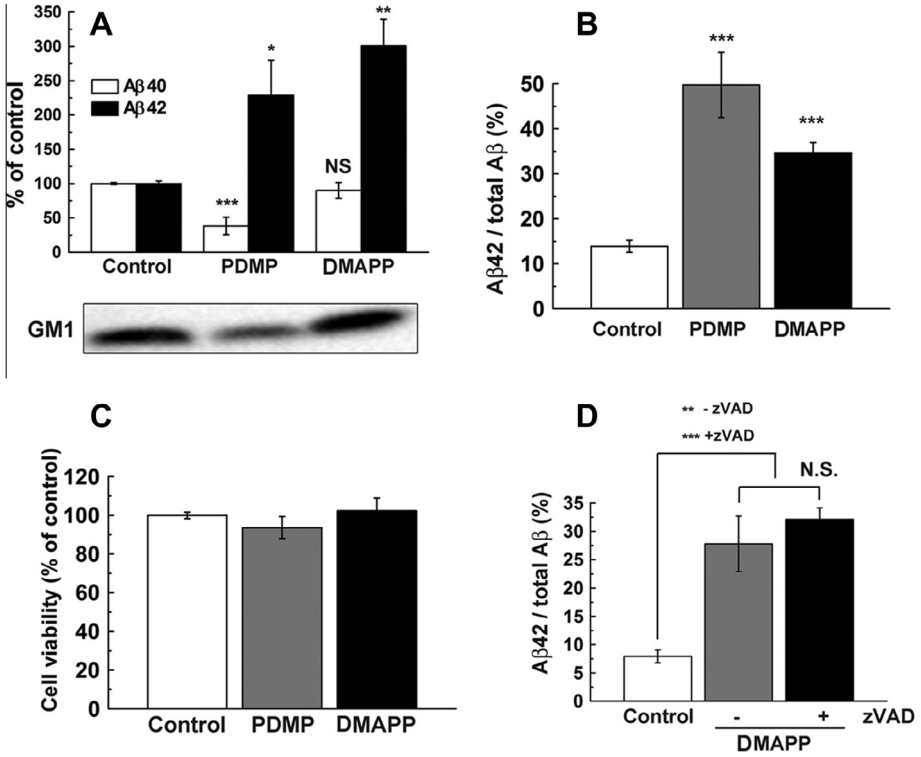

Fig. 2. Ceramide analogues modulate γ-secretase activity by a mechanism independent of apoptosis. (A, B) Neuro2a cells were treated with PDMP (10 μM) or DMAPP (10 μM) for 24 h, and secreted Aβ40 and Aβ42 were quantified. (A) Relative ratio of secreted Aβ40 and Aβ42 to vehicle treated control, respectively ($n = 4$, mean ± SEM; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; N.S.: not significant). Bottom panel represents the levels of GM1 ganglioside in the cell lysates. (B) Ratio of Aβ42 to total Aβ (%). (C) Cell viability analyzed by AlamarBlue ($n = 4$, mean ± SEM). (D) Neuro2a cells were treated with DMAPP (10 μM) in combination with or without zVAD-FMK (20 μM). The ratios of Aβ42 to total Aβ (%) are shown ($n = 4$, mean ± SEM).

4. Discussion

γ-Secretase is an intramembrane-cleaving protease that is comprised of Presenilin, Aph-1, Nicastrin, and Pen-2 [5,6]. We previously reported that the sphingolipid-related compounds FTY720 and KRP203 inhibited γ-secretase activity [18]. In the present study, we identified that the ceramide analogues PDMP and DMAPP increased the production of Aβ42 without affecting

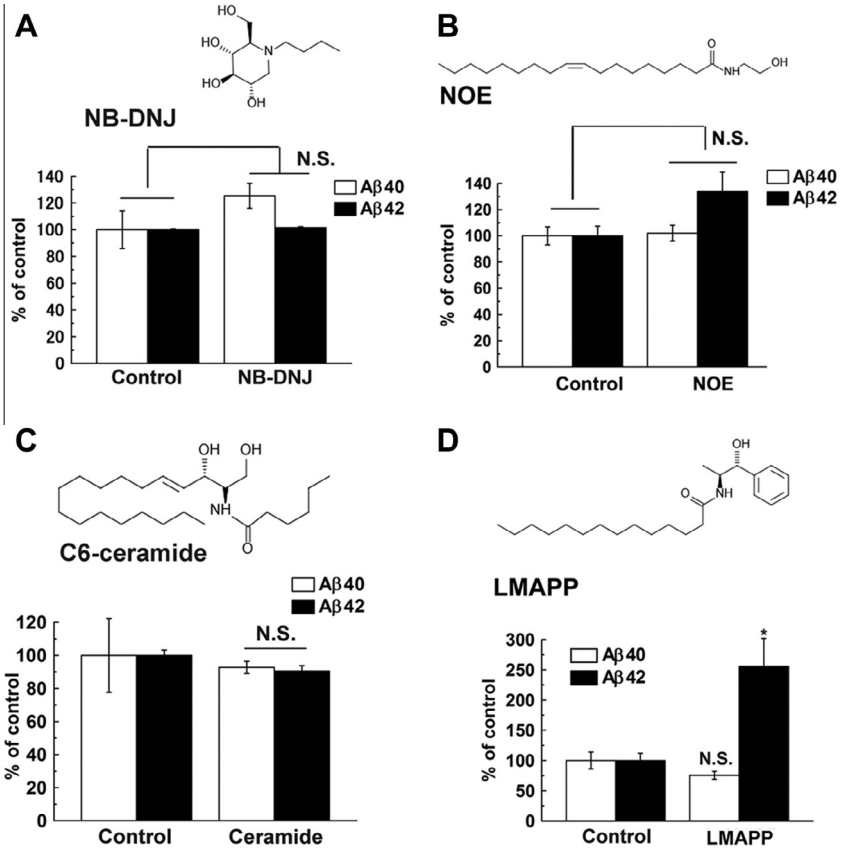

Fig. 3. Effect of non-ceramide analogue compounds and ceramide on Aβ secretion from Neuro2a cells. Neuro2a cells were treated with NB-DNJ (30 μM) (A), *N*-oleoylethanolamine (10 μM) (B), C6 ceramide (10 μM) (C), or LMAPP (10 μM) (D) for 24 h. Secreted Aβ was quantified by sandwich ELISA ($n = 4$, mean ± SEM; N.S.: not significant).

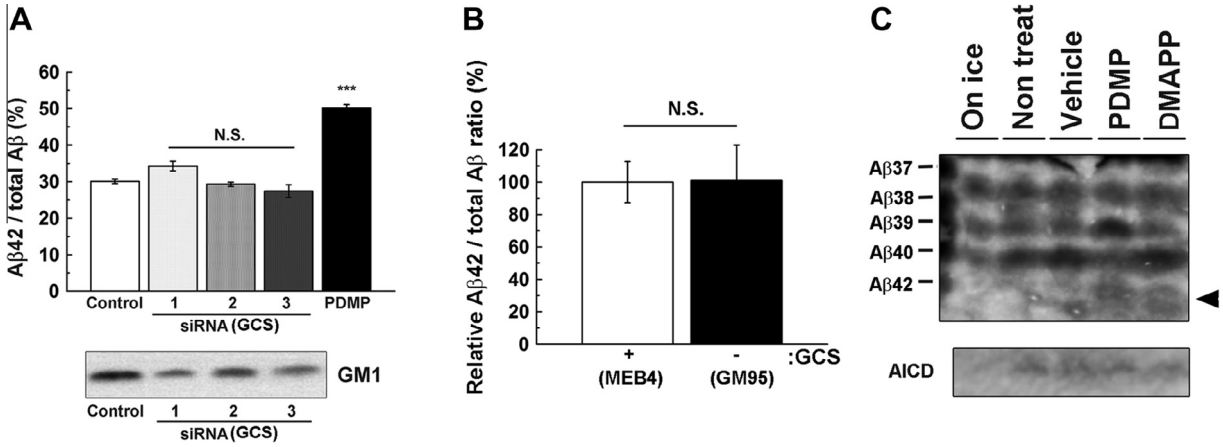

Fig. 4. Genetic modulation of ceramide metabolism did not modulate Aβ production. (A) HEK293 cells stably expressing the Swedish mutant of APP were transfected with siRNAs for GCS. After 48 h, the medium was replaced and the cells were further incubated for 24 h. As a control, cells were treated for 24hrs with PDMP (10 μM). The ratio of Aβ42 to total Aβ42 in the secreted Aβ ($n = 3$, mean ± SEM. $^{***}P < 0.001$) (top panel) and GM1 ganglioside levels in the cell lysates (lower panel) are shown. (B) The ratio of secreted Aβ42 to total secreted Aβ42 in a GCS-deficient cell line (GM95) and its parental control cell line (MEB4) transiently expressing SC100 ($n = 3$, mean ± SEM; N.S.: not significant). (C) Effect of PDMP (10 μM) and DMAPP (10 μM) on Aβ and AICD production in a cell-free assay using membranes from C99-expressing CHO cells. As control experiments, incubations at 4 °C (On ice), with (vehicle) or without vehicle (non treat) were performed. The arrowhead indicates the migration position of the Aβ42 peptide.

ceramide metabolism. Interestingly, LMAPP, the inactive stereoisomer of DMAPP, also increased Aβ42 production (Fig. 3D). These compounds affected the processing of direct γ-secretase substrates in cultured cells and cell free analyses (Fig. 4C). These data indicate that ceramide analogues can modulate γ-secretase to upregulate Aβ42 production.

Ceramide can also tightly interact with the other sphingolipids and cholesterols [36] to form microdomains in the lipid bilayer, surrounded by specific proteins. Increased levels of ceramide are known to alter the three-dimensional structure of enzymes residing in the membrane [37]. Previously, we reported that fenofibrate, a γ-secretase modulator that increases Aβ42 production, reduces

water accessibility of the catalytic pore. Thus it is possible that ceramide analogues alter the structure of the catalytic pore of γ-secretase. However, C6 ceramide treatment did not result in Aβ42 overproduction (Fig. 3), suggesting that the structure of ceramide analogues is distinct from that of native ceramides. Interestingly, the acyl chain length of lipids that correlates with membrane thickness was found to affect γ-secretase activity [38]. Likewise, the acyl chain length of ceramide analogues might be a key factor for γ-secretase activity modulation. Further analyses of the structural requirement of ceramide analogues for the modulation of γ-secretase activity are required.

Previous studies showed that PDMP increases the secreted form of APP and decreases total Aβ [39,40]. Although we focused on the modulatory function of PDMP on γ-secretase, differences in the time course, cell type, and source of APP should explain the discrepancies with our results. Nevertheless, our study highlights the importance of lipid microenvironments on γ-secretase activity, and sheds light on the molecular mechanism of Aβ42 overproduction in the brains of sporadic AD patients, in which altered ceramide metabolism has been implicated.

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