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Enhanced amyloid- β generation by γ -secretase complex in detergent-resistant membrane microdomains with reduced cholesterol levels

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Abstract

A neuropathologic hallmark of Alzheimer's disease (AD) is the presence of senile plaques that contain neurotoxic amyloid- β protein ($A\beta$) species, which are generated by the cleavage of APP by secretases such as the γ -secretase complex, preferentially located in detergent-resistant membrane (DRM) regions and comprising endoproteolysed amino- and carboxyterminal fragments of presenilin, nicastrin, anterior pharynx defective 1, and presenilin enhancer 2. Whereas some of familial AD patients harbor causative *PSEN* mutations that lead to more generation of neurotoxic $A\beta_{42}$, the contribution of $A\beta$ generation to sporadic/late-onset AD remains unclear. We found that the carboxyterminal fragment of presenilin 1 was redistributed from DRM regions to detergent-soluble membrane (non-DRM) regions in brain tissue samples from individuals with sporadic AD. DRM fractions from AD brain sample had the ability to generate significantly more $A\beta$ and had a lower cholesterol content than DRM fractions from nondemented control subjects. We further demonstrated that lowering the cholesterol content of DRM regions from cultured cells contributed to the redistribution of γ -secretase components and $A\beta$ production. Taken together, the present analyses suggest that the lowered cholesterol content in DRM regions may be a cause of sporadic/late-onset AD by enhancing overall $A\beta$ generation.

Introduction

Alzheimer's disease (AD) is characterized by a progressive loss of memory and cognitive functions with synaptic impairment and neurodegeneration. It is the most common neurological disorder in the aged population, and it is estimated that ~70% of patients with dementia have AD (1, 2). The pathological hallmarks of AD are senile plaques, composed primarily of amyloid- β protein ($A\beta$), and neurofibrillary tangles (NFT) comprising phosphorylated tau. Soluble $A\beta$ oligomers are thought to impair neuronal function and lead to neurodegeneration by interfering with synapses before the formation of NFT (3).

$A\beta$ is generated by the proteolytic processing of amyloid β -protein precursor (APP). α - or β -Secretase initially cleaves APP to produce amino-terminal extracellular/luminal fragments, sAPP α or sAPP β , respectively, and membrane-associated carboxy-terminal fragments, APP CTF α or CTF β , respectively (4, 5). APP CTF α/β is cleaved at the ϵ -site by the γ -secretase complex, a membrane-embedded multimeric aspartic protease comprising presenilin 1 or 2 (PS1 or PS2), nicastrin (NCT), anterior pharynx defective 1 (APH-1), and presenilin enhancer 2 (PEN-2). This releases the carboxy-terminal half of APP CTFs, APP intracellular domain (AICD), into the cytosol (6, 7) and secretes the amino-terminal half of APP CTF α/β , p3 and $A\beta$ from APP CTF α and CTF β respectively (4, 8). Following the primary ϵ -cleavage, further cleavage of the amino-terminal half of APP CTF α/β at multiple γ -sites occurs, and various neurotoxic species of $A\beta$ including $A\beta_{49}$, $A\beta_{46}$, $A\beta_{43}$, and ultimately $A\beta_{40}$, the major $A\beta$ species, are generated from APP CTF β (9). Alternative cleavage of CTF β at the minor ϵ -site results in $A\beta_{48}$, $A\beta_{45}$, $A\beta_{42}$, and, finally, $A\beta_{38}$, which does not aggregate and is not neurotoxic (10). It is known that active γ -secretase complex resides in membrane lipid raft domain and cleaves substrates in the lipid raft, which are rich in cholesterol and sphingolipids, and the lipid rafts including active γ -secretase complex are possible to isolate biochemically from cell and brain membranes as the detergent-resistant membranes (DRM) (11, 12).

One familial AD is associated with mutations in *PSEN1* and *PSEN2* encoding PS1 and PS2, respectively. These mutations impair this exopeptidase-like activity of the γ -secretase complex, halting degradation at the $A\beta_{42}$ species (10). Another familial AD

includes pathogenic mutations of *APP* and increases overall A β generation (13), leads to the production of longer aggregative and neurotoxic A β isoforms, such as A β 42 (14, 15), or facilitates A β aggregation (16). Notably, a mutation in *APP* that was shown to suppress A β generation (17) was found to facilitate β' -site cleavage of the protein by BACE1 (18). However, it is not clear if altered A β generation contributes to the pathology of sporadic/late-onset AD. Other factors, such as the A β clearance system or A β -degrading enzymes, have been implicated in these cases (19-21), whereas other reports suggest that γ -secretase activity is altered in the absence of pathogenic *PSEN* mutations (22-24).

The mechanism(s) underlying altered cleavage of APP substrates in the absence of *PSEN* mutations remains unknown. Experimental manipulations of the proportions of various membrane lipids were shown to alter the C-terminal speciation of A β (25, 26), and AD brain samples exhibit reduced cholesterol levels in DRM fractions (27). To elucidate the mechanisms contributing to the pathology of AD, we examined DRM samples from patients with sporadic AD and nonneuronal cells, and assayed the generation of A β by γ -secretase *in vitro*.

Results

Altered distribution of γ -secretase components and cholesterol content in DRM fractions from AD brain

The membrane lipid raft-like microdomain enriched in active γ -secretase complex can be isolated by a combination of the mild detergent CHAPSO with sucrose gradient centrifugation as a DRM (12, 28-31). We isolated DRM fractions from autopsy brain samples of patients with sporadic AD (n=12) and age-matched nondemented (n=10) subjects (subject information is described in **Table 1**), and immunoblotted for PS1 CTF, flotillin 1 (DRM resident protein), and calnexin (detergent-soluble non-DRM resident protein). Flotillin 1 was predominantly recovered in DRM fractions 4 and 5 including the interface between 5% and 35% sucrose, whereas smaller amounts were recovered along with calnexin in non-DRM fractions 9 and 10 (**Fig. 1a**), which almost coincide with previous isolation of DRM (12). Notably, PS1 CTF was largely recovered only from DRM fractions from control samples but was present in non-DRM fractions from AD

samples (**Fig. 1a**). Moreover, the recovery of PS1 CTF from DRM fractions of AD subjects was significantly lower than from control subjects ($P < 0.05$) (**Fig. 1b**), indicating an altered membrane localization of PS1 CTF in the brains of individuals with sporadic AD. These findings suggest that the active γ -secretase complex may disperse from DRM to non-DRM regions in sporadic AD subjects, including ApoE $\epsilon 4$ carriers (indicated with open symbols in **Fig. 1b**), who express the stronger risk of a late-onset AD (32).

We also examined the cholesterol content in our samples and found that levels were significantly lower in DRM fractions from AD brain samples ($P < 0.01$) (**Fig. 1c**), consistent with a previous report (27). Interestingly, DRM fractions from ApoE $\epsilon 4$ carriers (open symbols) typically had lower cholesterol levels among AD subjects, suggesting that ApoE $\epsilon 4$ may contribute to decreased cholesterol levels in DRM microdomains.

We also examined the cholesterol contents and the levels of γ -secretase components in total membrane fraction (P100) prior to the fractionation of DRM with CHAPSO and sucrose gradient centrifugation. Interestingly, the cholesterol contents of total membrane fraction were identical in brain samples of control and AD subjects (**Supplementary Fig. S1**). The γ -secretase components, PS1 CTF, PEN-2, and total NCT which composed of mature (~120 kDa) and immature (~100 kDa) NCT in total membrane fraction, did not show significant difference in their levels between control and AD subjects (**Supplementary Fig. S2**). Taken together, γ -secretase components are likely to redistribute into non-DRM from DRM according to the decreased cholesterol level in DRM.

Altered A β production ability in DRM fractions from AD brain

To determine whether DRM fractions from AD subjects had an altered ability to generate A β , we performed an *in vitro* γ -secretase assay (31). DRM fractions containing the same amounts of protein were incubated with a purified FLAG-tagged APP CTF β (C99-FLAG) substrate. Enzyme-linked immunosorbent assays revealed that A $\beta 40$ and A $\beta 42$ generation was significantly elevated in DRM fractions from AD brains compared with those from control subjects ($P < 0.01$) (**Fig. 2a and b**). Samples from individuals that were carriers of ApoE $\epsilon 4$ (open symbols) tended to have larger amounts of A $\beta 40$ and A $\beta 42$. The ratio of A $\beta 42$ to A $\beta 40$ was slightly but significantly higher in AD samples

than in controls ($P < 0.01$) (**Fig. 2c**), consistent with that observed in familial AD subjects harboring pathogenic *PSEN* mutations (**9, 33, 34**). These results indicate that AD subjects who show aberrant membrane distribution of γ -secretase produce greater amounts of A β and neurotoxic A β species.

We also asked the levels of endogenous APP, APP CTFs and BACE1 in the DRM fractions from AD subjects prepared with CAPSO (**Supplementary Fig. S3**). Recoveries of APP and BACE1 in DRM fractions are almost identical without a statistical significance. The result suggests that the levels of endogenous substrate APP and primary APP cleaving enzyme BACE1 don't contribute for A β generation in an in vitro γ -secretase activity assay, although we could not determine the levels of endogenous APP CTFs, direct substrates to γ -secretase, in DRM fractions because of its too low levels to quantify. To perform further biochemical analysis for relationship of cholesterol level with γ -secretase distribution and A β generation ability in DRM, we used DRM of cells, in which the membrane cholesterol level can be experimentally manipulated.

Cholesterol depletion alters γ -secretase distribution in DRM fractions

To further examine the relationship between cholesterol content and the distribution of γ -secretase components in cellular membranes, we treated HEK293 cells with methyl- β -cyclodextrin (M β CD) to deplete cholesterol or with water-soluble cholesterol (cholesterol-saturated M β CD) to enhance the cholesterol content. DRM fractions from cells treated with M β CD had cholesterol levels ~50% lower than nontreated control cells ($P < 0.001$), whereas levels were significantly increased in cells treated with cholesterol-saturated M β CD ($P < 0.01$) (**Fig. 3a**). Membrane microlocalization of γ -secretase components PS1 CTF, NCT and PEN-2 was examined by immunoblotting (**Fig. 3b**), in which APH-1 was not examined because we could not obtain the antibody. DRM fraction including the interface between 5% and 35% sucrose (fractions 3 and 4) and non-DRM fraction (fraction 7) are confirmed with the residency of respective marker protein, flotillin1 and calnexin. Immunoblotting analysis of the various fractions revealed that distribution patterns of calnexin, flotillin 1, and PS1 CTF from cells treated with M β CD were similar to those for AD tissue samples (**Fig. 3b**). We further quantified the percentages of PS1 CTF, NCT, and PEN-2 recovered from DRM fractions and found that the amounts were significantly decreased in cells treated with M β CD compared with that

in untreated control cells ($P < 0.001$) (**Fig. 3c**). By contrast, the recovery of these γ -secretase components was significantly higher in cells treated with cholesterol-saturated M β CD than in controls (all $P < 0.05$).

We examined again the cholesterol contents and the levels of γ -secretase components in total membrane fraction (P100) of the HEK293 cells prior to the fractionation of DRM with CHAPSO and sucrose gradient centrifugation. Cholesterol levels, slightly but significantly, decreased in P100 membrane fraction of cells treated with M β CD and increased in that of cells treated with cholesterol-saturated M β CD (**Supplementary Fig. S4**). However, the levels of γ -secretase components, PS1 CTF, PEN-2, and total NCT (mature plus immature NCT) in total membrane fraction were almost same among cells with or without treatments (**Supplementary Fig. S5**). Although slight changes of cholesterol levels in total crude membrane of HEK293 cells were observed by the treatments with reagents, γ -secretase components are likely to redistribute into non-DRM from DRM by the decreased cholesterol levels in DRM as observed in human brain samples.

Correlation between the amount of cholesterol and residency of γ -secretase components in DRM was examined (**Fig. 4**). Recovery of PS1 CTF, NCT and PEN-2 was significantly correlated with the amount of cholesterol in DRM (all $P < 0.0001$). These results strongly suggest that the amount of cholesterol in the membrane influences the distribution of the γ -secretase complex.

Another lipid component enriched in membrane lipid raft region is sphingolipids. We also examined the recovery of γ -secretase components in DRM using two types of mutant cells which showed reduced sphingolipids (**Supplementary Fig. S6**). LY-A cells are defective in sphingomyelin synthesis due to aberrant intracellular transport of ceramide, and LY-B cells are defective in the enzyme to biosynthesis sphingolipid species (35). Therefore, these mutant cells express the reduced level of sphingolipid respective ~40% (LY-A) and ~15% (LY-B) compared to their parental CHO-K1 cell. Membrane microlocalization of γ -secretase components PS1 CTF, NCT and PEN-2 was examined by immunoblotting as described in Fig. 3. Distribution patterns of γ -secretase components and calnexin and flotillin 1 from mutant cells were almost similar to those from parental wild-type CHO cells. Furthermore, their revertant cells, LY-A/hCERT and LY-B/cLCB1, also showed the same distribution of γ -secretase components when compared to mutant

cells. Taken together, no significant changes were observed in the recovery of PS1 CTF, NCT and PEN-2 in DRM among wild-type, mutant and revertant cells. Therefore, we focused our further analysis for A β generation assay on cholesterol level in DRM.

Cholesterol depletion alters A β production in DRM fractions

We next examined A β generation from C99-FLAG in DRM fractions from HEK293 cells treated with M β CD. The levels of γ -secretase components in the DRM fractions used in the A β generation assay were first assessed by immunoblotting (**Fig. 5a**). The levels of flotillin 1 did not differ significantly among DRM fractions from control cells, cells treated with M β CD, and cells treated with cholesterol-saturated M β CD (**Fig. 5b**). However, cells treated with M β CD had significantly lower amounts of PS1 CTF, NCT, and PEN-2, whereas cells treated with cholesterol-saturated M β CD had significantly higher levels ($P < 0.05$ or $P < 0.01$ vs. control) (**Fig. 5b**). Using these DRM samples, A β generation assay was performed (**Fig. 6**). Notably, the production of A β 40, A β 42, and A β 38 was significantly higher in DRM of M β CD-treated cells ($P < 0.01$ or $P < 0.001$ vs control), whereas production was significantly lower in DRM of cells treated with cholesterol-saturated M β CD ($P < 0.05$ or $P < 0.01$ vs control) (**Fig. 6a**). However, the ratios of A β 42 to A β 40 or A β 38 were not significantly different among the treatments (**Fig. 6b**).

Again the levels of endogenous APP, APP CTFs and BACE1 in the DRM fractions from cells treated with or without M β CD and cholesterol-saturated M β CD were examined (**Supplementary Fig. S7**). Recoveries of APP, APP CTF and BACE1 in DRM fractions decreased by the treatment with M β CD but increased by treatment with cholesterol-saturated M β CD significantly. Using same DRM samples we tried to quantify A β 40 generated endogenously in the in vitro γ -secretase assay without exogenous substrate. The levels are almost identical to background level which is a value of assay including γ -secretase inhibitor (10 μ M DAPT, 3,5-difluorophenylacetyl)-Ala-Phg-OBu) (data not shown). Because the A β generation in the in vitro γ -secretase assay with exogenous substrate C99-FLAG increased in DRM from cells treated with M β CD, but decreased in that with cholesterol saturate M β CD (**Fig. 6**), endogenous protein

components except for γ -secretase components did not contribute for A β generation largely in this in vitro γ -secretase activity assay.

Correlation between the amount of cholesterol and A β generation of DRM was examined (**Fig. 7**). Ability to generate A β in DRM showed a significant inverse correlation for A β 40 ($P < 0.0001$), A β 42 ($P < 0.0001$) and A β 38 ($P < 0.0001$) (**Fig. 7a**), but no correlation was observed between A β 42/40 and A β 42/38 ratios and cholesterol amount (**Fig. 7b**). These data suggest that the amount of cholesterol in DRM also influences the production of all A β species.

Finally, we analyzed the correlation of cholesterol levels in DRM with AD pathology, senile plaque and neurofibrillary tangle (NFT) scores, in human subjects (**Table 1**). Interestingly, both senile plaque and NFT scores showed an significant inverse correlation with cholesterol level of DRM (**Fig. 8**). This analysis supports an idea that the decreased cholesterol level in DRM may facilitate AD pathology along with increased A β generation.

Discussion

Whereas the factors leading to familial AD are largely known, the causes of sporadic/late-onset AD are still unclear. Various hypotheses have been proposed involving impaired A β clearance by enzymatic degradation, cellular uptake, or vascular removal (**19, 36-38**). Our previous studies on the metabolism and function of APP (**5, 18, 39-44**) and evidence that the cleavage of γ -secretase substrates is altered in individuals with sporadic AD without known causative genetic mutations (**22-24**) led us to hypothesize that A β generation is altered in these patients. Indeed, our data show that brain tissue samples from patients with sporadic AD have an ability to generate greater amounts of neurotoxic A β species.

We also found that the components of active γ -secretase redistribute from DRM regions to non-DRM regions in association with a lowered cholesterol level. These findings are consistent with the depletion of cholesterol and altered levels of several types of gangliosides in DRM fractions of AD brains reported previously (**27**). However, studies with cholesterol-lowering drugs suggest that lowering the total cholesterol level reduces the levels of A β in cells and *in vivo* (**45**). Although the role of cholesterol in the regulation of γ -secretase activity in DRM regions remains unclear, recent structural

analyses suggest that the membrane lipid microenvironment influences the structure and function of γ -secretase complex (46). Our *in vitro* data support this, as the distribution of γ -secretase complex components between DRM and non-DRM regions was altered by modifying the cellular cholesterol level. Moreover, the DRM fractions from ApoE ϵ 4 carriers with AD tended to have lower levels of cholesterol and the ability to generate more A β . Altogether, this may be important evidence that changing the cholesterol level in DRM regions influences A β generation by γ -secretase complexes.

Although the decrease of cholesterol content in DRM fractions corresponded to enhanced overall production of A β in both AD tissues and cultured cells, there were slight differences between the results from both experiments. For example, the increased A β 42/A β 40 ratio in AD tissue samples was not observed in the cholesterol-depleted cells, despite similar increases in both A β species. There may be another factor(s) in DRM regions of brain tissue that alter A β generation. Nevertheless, the overall enhanced production of A β with lower cholesterol levels in DRM despite the decrease in γ -secretase components may be one of the pathogenic causes of sporadic/late-onset AD. It is possible that an increase in membrane fluidity as result of reduced amounts of cholesterol facilitates interactions between APP CTF and the remaining γ -secretase complex in DRM regions. However, further investigations are needed to elucidate the precise mechanisms for enhanced A β generation in DRM regions with lower cholesterol levels.

Our data also raise the question as to whether the redistribution of γ -secretase components from DRM regions results in enhanced γ -secretase activity in non-DRM regions. Although the biochemical non-DRM fraction prepared in this study is a solution including solubilized membrane components that contain the five membrane protein components (amino- and carboxyterminal fragments of PS1, NCT, PEN-2, and APH-1) of the large γ -secretase complex, it is believed that these solubilized components cannot form a structure with full enzyme activity (31). However, if A β generation is increased in non-DRM regions of innate membranes from AD tissues as in cholesterol-depleted cells, this would support our conclusion that lower cholesterol levels increase overall A β generation.

Although we cannot rule out other possible factors for altered A β generation,

the present results suggest that dispersion of active γ -secretase components from DRM regions by result lowered cholesterol levels may contribute to the pathogenesis of sporadic AD with the upregulated production of A β .

Materials and Methods

Antibodies

These studies utilized antibodies against calnexin (Enzo Life Sciences, Farmingdale, NY, USA), flotillin 1 (BD Biosciences, San Jose, CA, USA), PEN-2 (Merck Millipore, Darmstadt, Germany), NCT (Sigma-Aldrich, St. Louis, MO, USA), BACE1 (Cell Signaling Technology, Danvers, MA USA), PS1 (G1L3 antibody described previously (47, 48)), and APP cytoplasmic region (described previously (49)).

Cell culture

HEK293 cells were maintained in DMEM (FUJIFILM Wako Pure Chemical, Osaka, Japan) including 5% (v/v) heat-inactivated fetal bovine serum (MP Biomedicals, Solon, OH, USA) and antibiotic-antimycotic solution (70 μ g/ml penicillin, 100 μ g/ml streptomycin, 2.5 μ g/ml amphotericin B; FUJIFILM Wako Pure Chemical) at 37°C in a humidified cell culture chamber under 5% CO₂.

For cholesterol depletion, HEK293 cells cultured in serum-free DMEM were incubated with or without 10 mM M β CD (FUJIFILM Wako Pure Chemical) for 1 h. For cholesterol incorporation, the cells were incubated for 30 min in serum-free DMEM containing 0.1 mM water-soluble cholesterol (Sigma-Aldrich), which consisted of M β CD containing cholesterol. The cells were harvested for analysis.

Sf9 cells were maintained with PSFM-J1 medium (FUJIFILM Wako Pure Chemical) including 2.5% (v/v) fetal bovine serum, 100 μ g/ml penicillin, and 100 μ g/ml streptomycin (Gibco of Thermo Fisher Scientific, Waltham, MA, USA) at 27°C in a cell culture chamber.

Chinese hamster ovary (CHO) cell lines were supplied from Dr. Nishijima, cultured as described (35) and used for assay (**Supplementary Fig. S6**).

Human brain tissue samples

Frozen tissues from the frontal and temporal cortices of 12 AD patients and 10 nonneurological disease control subjects were obtained from the Brain Bank for Aging Research, Tokyo Metropolitan Institute of Gerontology (Tokyo, Japan). Ethical boards at Hokkaido University Faculty of Pharmaceutical Sciences approved the use of human samples and the Medical Science Review Board of Shiga University of Medical Science approved the study protocol. AD was clinically diagnosed according to criteria of the National Institute of Neurological and Communicational Disorders and Stroke–AD and Related Disorders Association. Subject information is provided in **Table 1**. Senile plaque and NFT staging were according to Braak's criteria (**50, 51**). Senile plaques and NFT staging are 0, 0.5 or 1 and 1 or 2 respectively in normal subjects, while those are respective 3 and 4, 5 or 6 in AD patients.

Preparation of DRM fractions

Cells ($\sim 10^8$) harvested from six 10 cm plates were lysed in 1.8 ml TNE buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA) by passing them ten times through a 30-gauge needle on ice. Unbroken organelles and nuclei were removed by centrifugation at $3,000 \times g$ for 10 min at 4°C. The supernatant was further centrifuged at $100,000 \times g$ (TLA55 rotor; Beckman Coulter, Brea, CA, USA) for 1 h. The precipitated membrane fraction (P100) was resuspended in 500 μ l TNE, and the detergent, 1% (w/v) CHAPSO (3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonate; Dojindo, Kumamoto, Japan), was added. DRM and non-DRM fractions were prepared by discontinuous sucrose gradient centrifugation. Samples (500 μ l) were mixed with equal volumes of 85% sucrose in TNE, layered in a tube containing a discontinuous gradient solution of 5% (3 ml), 35% (3 ml), and 42.5% (1 ml) sucrose in TNE, and centrifuged at $260,000 \times g$ (SW41Ti rotor; Beckman Coulter) for 20 h. Seven fractions (1 ml) were collected starting from the top (fraction 1) of the ultracentrifuge tubes. DRM is recovered from fractions including the interface between 5% and 35% sucrose, which is confirmed by flotillin 1 resident (**12**).

Human brain samples (35–40 mg tissue weight) were lysed in an 8-fold volume of TNE buffer with a Dounce homogenizer (Dounce tissue grinder, tight; 30 times) and then

passed 30 times through a 27-gauge needle on ice. Tissue residues were removed by centrifugation at $3,000 \times g$ for 10 min at 4°C, and the supernatant was further centrifuged at $100,000 \times g$ in a TLA55 rotor for 1 h. The P100 fraction was resuspended in 1 ml TNE with 1% (w/v) CHAPSO. DRM fractions of human samples were prepared as described above with an exception of fraction size (5% [4 ml], 35% [4 ml], and 42.5% [2 ml] sucrose in TNE), with ten fractions collected starting from the top (fraction 1) of the ultracentrifuge tubes. DRM is again recovered from fractions including the interface between 5% and 35% sucrose (12). Proteins in the respective fractions were analyzed by immunoblotting with the above-indicated antibodies and quantitated with a LAS-4000 mini imager (FUJIFILM, Tokyo, Japan). Cholesterol contents of DRM samples were quantitated using the LabAssay cholesterol kit (FUJIFILM Wako Pure Chemical).

***In vitro* γ -secretase assay with DRM from cells and human brain tissue**

C99 with a FLAG tag at the carboxyl terminus and signal peptide at the amino-terminal region was inserted into the pFASTBACTM1 vector (Invitrogen, Carlsbad, CA, USA) as described previously (52). Sf9 cells were infected with the recombinant baculovirus and cultured in the presence of 20 μ M GM6001 (Sigma-Aldrich) to suppress α -secretase-like cleavage of C99-FLAG. C99-FLAG overexpressed in Sf9 cells was solubilized in a lysis buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% [v/v] Nonidet P-40) containing protease inhibitors (5 μ g/ml each of chymostatin, leupeptin, and pepstatin A [Peptide Institute Osaka, Japan]) and then immunoaffinity-purified with anti-FLAG M2-agarose beads (Sigma-Aldrich). The quantity and purity of C99-FLAG were assessed by staining with Coomassie brilliant blue R-250 following separation by gel electrophoresis, and the purified product was used as the substrate in a γ -secretase assay as described previously (31).

DRM fractions from cells were suspended in a 3-fold volume of buffer H (20 mM HEPES [pH 7.4], 150 mM NaCl, 5 mM EDTA, 10% [v/v] glycerol) including 0.25% (w/v) CHAPSO and protease inhibitors, and centrifuged at $140,000 \times g$ in a TLA55 rotor for 1 h at 4°C. The resultant precipitates were resuspended in the same buffer without protease inhibitors and mixed well by passing 10 times through a 30-gauge needle. The purified C99-FLAG substrate (26 nM final concentration) was added to this suspension

and incubated for 2 h at 37°C with or without 10 μ M *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-(*S*)-phenylglycine *t*-butyl ester (Sigma-Aldrich), a γ -secretase inhibitor.

The DRM fractions from human brain tissue were suspended in a 4-fold volume of buffer C (50 mM PIPES [pH 7.0], 0.25 M sucrose, and 1 mM EGTA) including 0.25% (w/v) CHAPSO and protease inhibitors, and centrifuged at $100,000 \times g$ for 1 h at 4°C. The resultant precipitates were resuspended in a 4-fold volume of the same buffer without protease inhibitors and mixed well by passing 10 times through a 30-gauge needle. The purified C99-FLAG substrate (5 nM final concentration) was added to this suspension and incubated for 4 h at 37°C.

The membrane suspensions were centrifuged at $10,000 \times g$ for 10 min, and the amounts of A β 38, A β 40, and A β 42 in the supernatants were quantified with commercial enzyme-linked immunosorbent assay kits (IBL, Co. Ltd., Fujioka Japan). A β values were normalized against the amount of total protein. Background endogenous A β levels were obtained from the samples treated with the γ -secretase inhibitor, and the levels were negligible.

Statistical analysis

Data are expressed as the means $\pm$ SEs. Statistical differences were assessed using Student's *t*-test or one-way ANOVAs combined with Dunnett's test for multiple comparisons (GraphPad Prism 4 software). Statistical analysis was performed by using the Pearson's correlation coefficient test for correlation analysis. A *p* value of <0.05 was considered significant. No sample size calculation and no tests for normal distribution or outliers were performed. The study was not pre-registered.

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Conflict of Interest

All authors declare no conflict of interest.

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Figure legends

Figure 1. Altered distribution of PS1 CTF in human brain tissue membranes.

(a) Isolation of DRM and non-DRM fractions. One-milliliter fractions including DRM (4 and 5) and non-DRM (9 and 10) from human brain tissues from nondemented age-matched control and AD subjects were collected, and samples (24 μ l/lane) were analyzed by immunoblotting to determine the localization of PS1 CTF, calnexin (non-DRM resident), and flotillin 1 (DRM resident). Representative blots are shown. The numbers indicate molecular size (kDa).

(b) Recovery of PS1 CTF from DRM fractions from control and AD subjects. Recovery was quantitated from band densities (panel a), and the recovery from DRM fractions (4 and 5) is indicated.

(c) Cholesterol contents in DRM fractions. Cholesterol levels were standardized with protein contents.

In panels b and c, open symbols indicate ApoE ϵ 4 carriers. Statistical analysis was performed using Student's *t* tests ($n = 12$ AD, 10 control; $*P < 0.05$; $**P < 0.01$). Data are shown as means $\pm$ SEMs.

Figure 2. A β production ability in DRM fractions from brain tissues of AD subjects and controls.

(a-b) Generation of A β with DRM. γ -Secretase activity for the generation of A β in DRM fractions from human brain (4 μ g protein/assay) was examined *in vitro* using an exogenous substrate, C99-FLAG. Generation of A β 40 (a) and A β 42 (b) was compared between AD and control samples.

(c) A β 42/40 ratio is shown.

Open symbols indicate ApoE ϵ 4 carriers. Statistical analysis was performed using Student's *t* tests ($n = 12$ AD, 10 control; $**P < 0.01$). Data are shown as means $\pm$ SEMs.

Figure 3. Cholesterol levels and altered distribution of γ -secretase components in DRM fractions from HEK293 cells treated with or without M β CD and cholesterol-saturated M β CD.

(a) Cholesterol levels in DRM fractions. Cells were treated with M β CD (10 mM), water-soluble cholesterol (0.1 mM), or without reagents (control). Cholesterol contents were standardized with protein amounts. Statistical analysis was performed using Dunnett's

multiple comparison test ($n = 8$; $**P < 0.01$; $***P < 0.001$).

(b) Characterization of γ -secretase components in DRM and non-DRM fractions. DRM (3 and 4) and non-DRM (7) fractions were prepared from HEK293 cells treated as in panel a. Membrane fractions (10 μ l) were analyzed by immunoblotting to determine the presence of PS1 CTF, NCT (closed arrowhead, mature NCT; open arrowhead, immature NCT), and PEN-2 along with calnexin (non-DRM resident) and flotillin 1 (DRM resident). Representative blots are shown. The numbers indicate molecular size (kDa).

(c) Recovery of PS1 CTF, NCT, and PEN-2 from DRM fractions of cells treated with or without reagents as in panel a. Proteins were quantitated from bands densities on blots shown in panel b, and the recoveries in DRM fractions (3 and 4) are indicated. Statistical analysis was performed using Dunnett's multiple comparison test ($n = 5$; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$). Data are shown as means $\pm$ SEMs.

Figure 4. Correlation of γ -secretase residency with cholesterol level in DRM.

The correlation between recovery of PS1 CTF (a), NCT (b) and PEN-2 (c) and cholesterol levels are shown (all $P < 0.0001$, $n = 15$) with results of Fig. 3. Statistical analysis was performed by using the Pearson's correlation coefficient test.

Figure 5. Levels of γ -secretase components in DRM fractions used in A β production assay.

(a) Immunoblot analysis of γ -secretase components in DRM fractions used for *in vitro* γ -secretase assays in Fig. 5. Equal amounts (10 μ l) of DRM fractions prepared from cells treated with M β CD (10 mM), water-soluble cholesterol (0.1 mM), and without reagents (control) were analyzed by immunoblotting for PS1 CTF, NCT (closed arrowhead, mature NCT; open arrowhead, immature NCT), PEN-2, and flotillin 1. Representative blots are shown. The numbers indicate molecular size (kDa).

(b) Quantitation of flotillin 1, PS1 CTF, NCT, and PEN-2 from bands densities using blots in panel a; relative ratios to control (1.0) are indicated. Statistical analysis was performed using Dunnett's multiple comparison test ($n = 5$; $*P < 0.05$; $**P < 0.01$; n.s., not significant). Data are shown as means $\pm$ SEMs.

Figure 6. A β production ability in DRM fractions from HEK293 cells treated with or without M β CD and cholesterol-saturated M β CD.

- (a) Generation of A β from C99-FLAG in DRM fractions described in legends for Fig. 3 and 4. A β 40 (left), A β 42 (middle), and A β 38 (right) were quantitated. Statistical analysis was performed using Dunnett's multiple comparison test ($n = 8$; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; n.s., not significant). Data are shown as means $\pm$ SEMs.
- (b) A β 42/A β 40 and A β 42/A β 38 ratios were calculated using net values from data in panel a. Statistical analysis was performed using Dunnett's multiple comparison test.

Figure 7. Correlation of A β generation with cholesterol level in DRM.

- (a) The correlation between A β generation and cholesterol levels are shown (A β 40, $P < 0.0001$; A β 42, $P < 0.0001$; A β 38, $P < 0.0001$; $n = 24$) with results of Fig. 6.
- (b) The correlation between A β ratio and cholesterol levels are shown (A β 42/40, $P = 0.9481$; A β 42/38, $P = 0.8299$). Statistical analysis was performed by using the Pearson's correlation coefficient test.

Figure 8. Correlation of senile plaque and NFT scores with cholesterol level in DRM.

- (a) The correlation between senile plaque stage and cholesterol levels are shown ($*P=0.0414$).
- (b) The correlation between NFT stage and cholesterol levels are shown ($*P=0.035$). Statistical analysis was performed by using the Pearson's correlation coefficient test.

Table 1. Summary of subject information

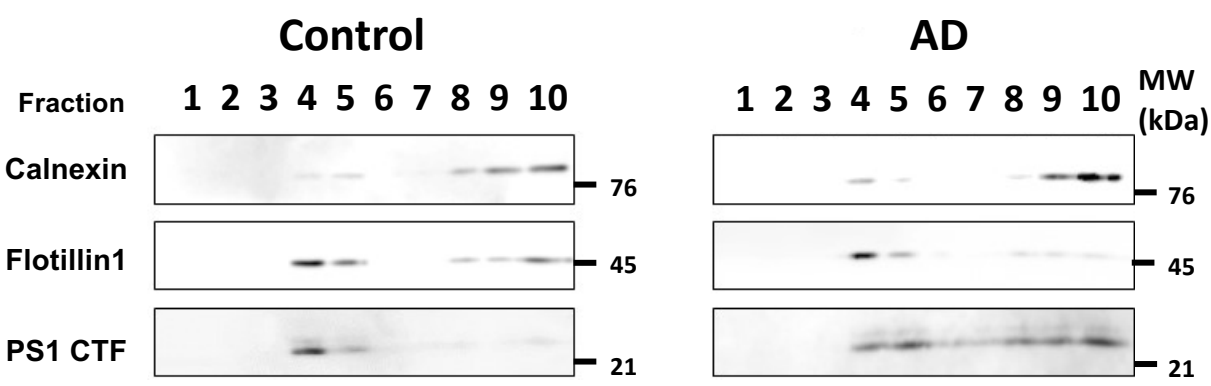
	Age	Gender	ApoE	CDR	Senile plaque	NFT
Normal	81	M	2/3	0	0	1
Normal	84	F	3/3	0	1	1
Normal	78	F	3/3	0	1	2
Normal	82	F	3/3	0	0.5	1
Normal	82	F	3/3	0	0	2
Normal	80	M	3/3	0	0.5	2
Normal	82	M	3/3	0	1	1
Normal	78	M	2/3	0	0	1
Normal	78	M	3/3	0	0	1
Normal	82	M	3/3	0	1	1
AD	81	F	3/3	≥ 1	3	5
AD	79	F	3/3	1	3	5
AD	74	M	3/4	1	3	5
AD	84	M	3/3	2	3	4
AD	76	M	2/3	3	3	6
AD	82	F	3/3	3	3	6
AD	82	F	3/3	3	3	5
AD	82	F	3/3	3	3	5
AD	84	M	3/4	3	3	5
AD	75	M	4/4	3	3	6
AD	81	M	3/3	1 or 2	3	6
AD	70	M	4/4	≥ 1	3	4

Normal, non-demented subject; AD, Alzheimer's disease; M, men; F, female; CDR, clinical dementia rating; NFT, neurofibrillary tangle. ApoE genotype: 2, $\epsilon 2$; 3, $\epsilon 3$; 4, $\epsilon 4$.

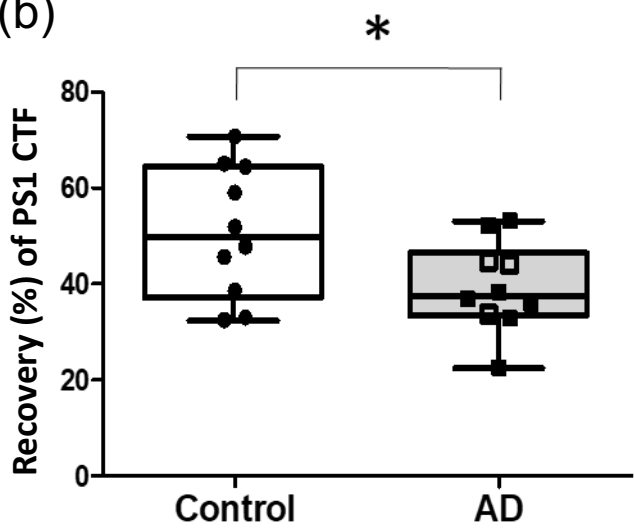
Abbreviations

A β , amyloid- β protein; AD, Alzheimer's disease; APH-1, anterior pharynx defective 1; APP, amyloid precursor protein; CTF, carboxy-terminal fragment; DRM, detergent-resistant membrane; M β CD; methyl- β -cyclodextrin; NCT, nicastrin; NFT, neurofibrillary tangles; PEN-2, presenilin enhancer 2; PS1/2, presenilin 1/2; Apo E, apolipoprotein E.

(a)



(b)



(c)

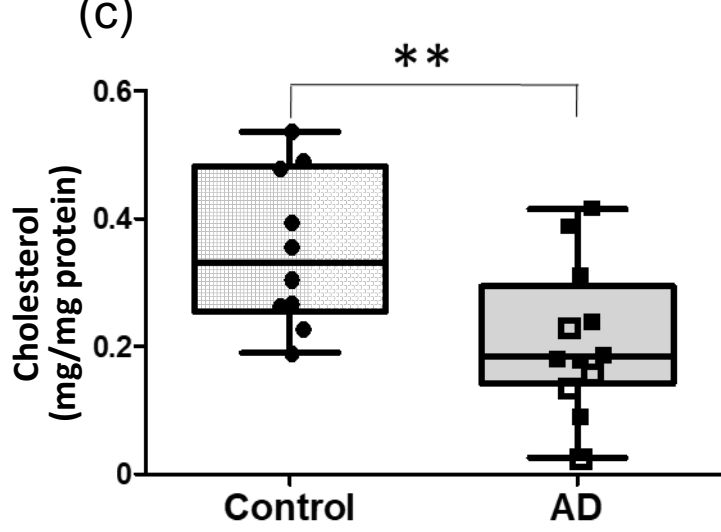


Fig.1

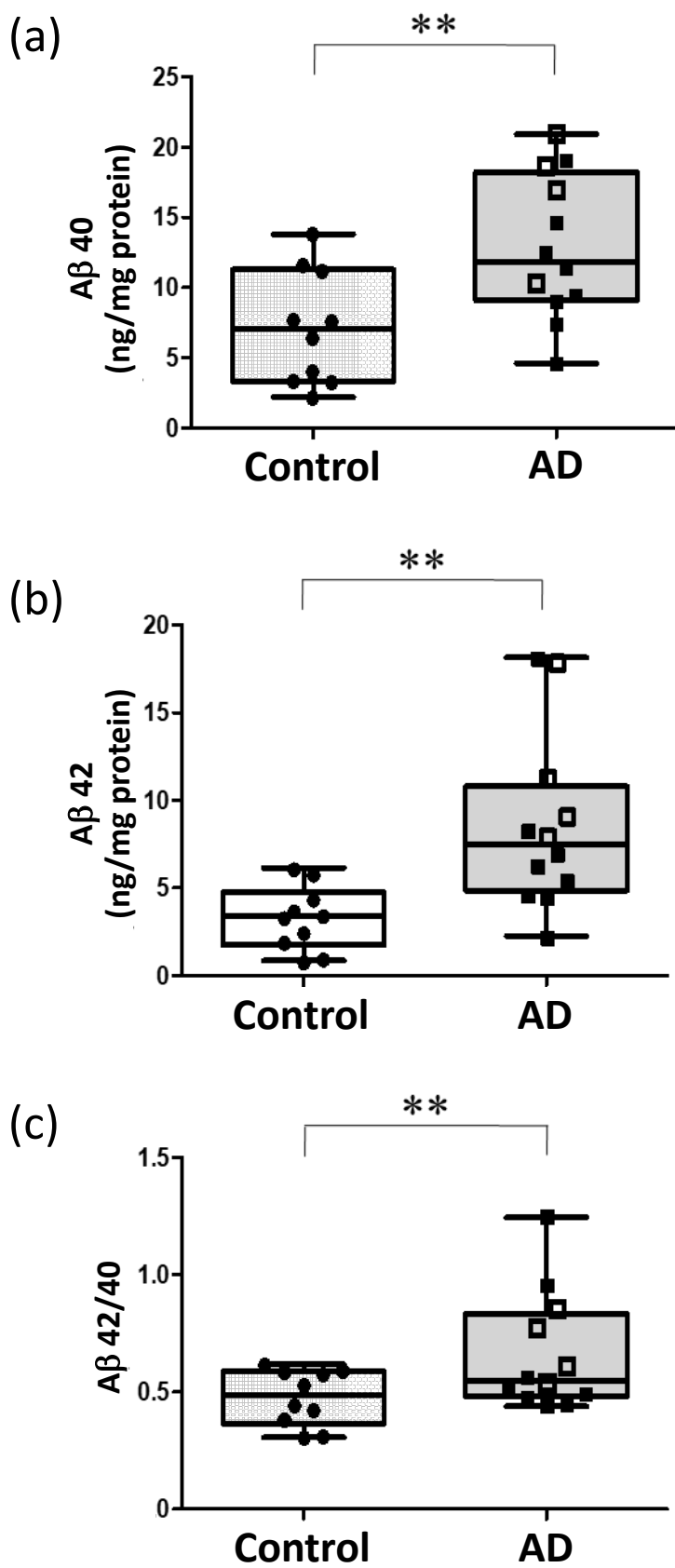


Fig. 2

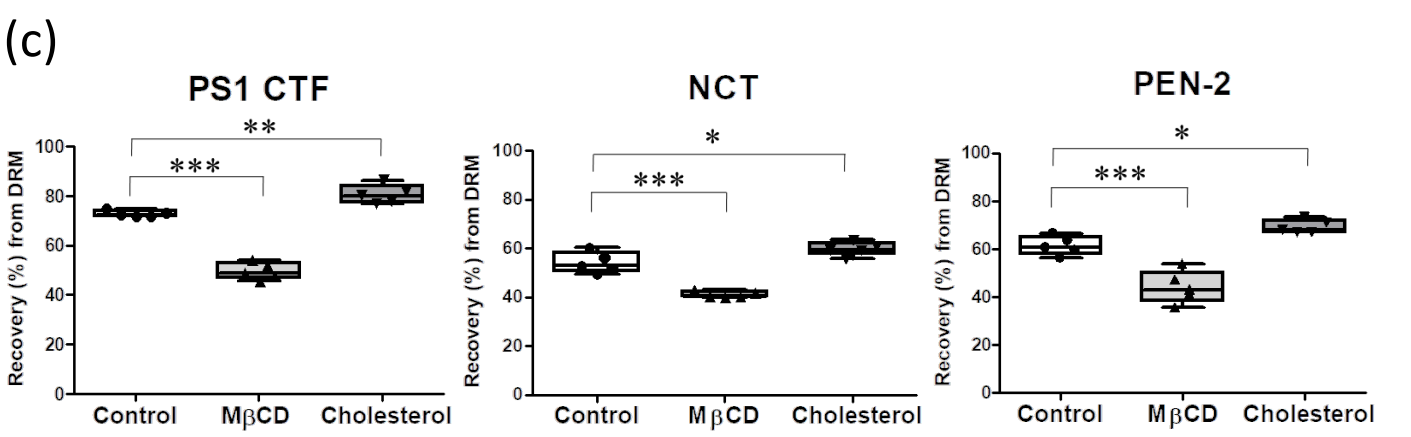
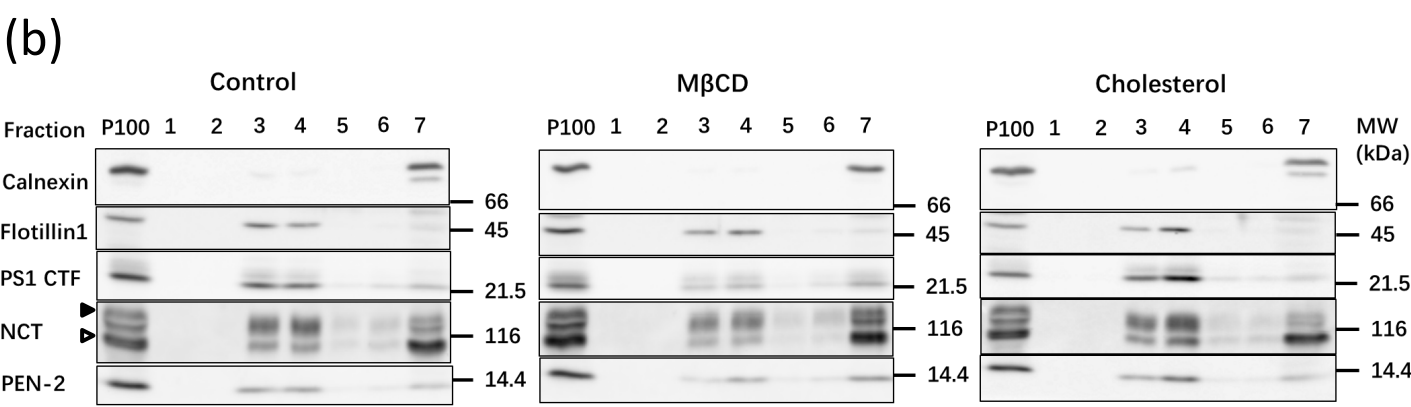
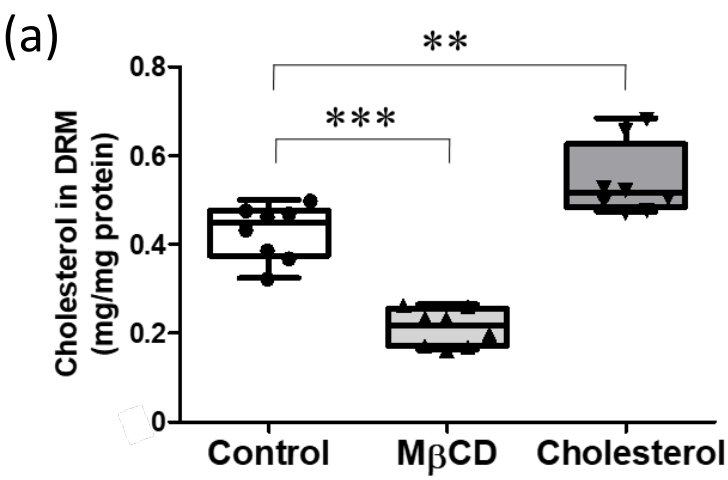


Fig. 3

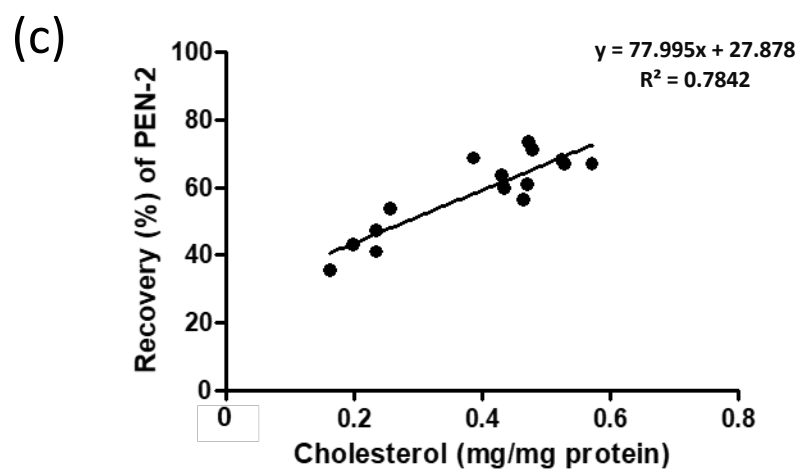
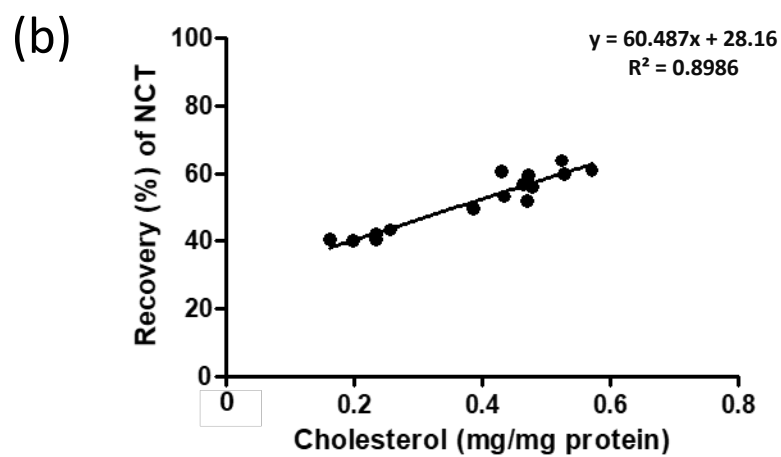
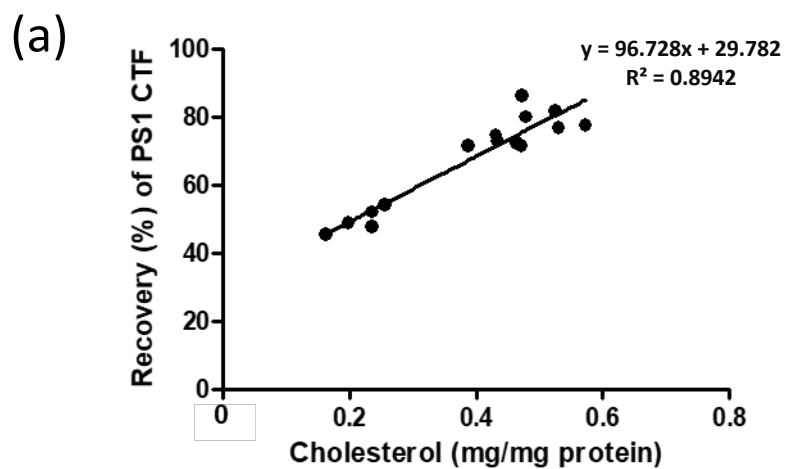


Fig. 4

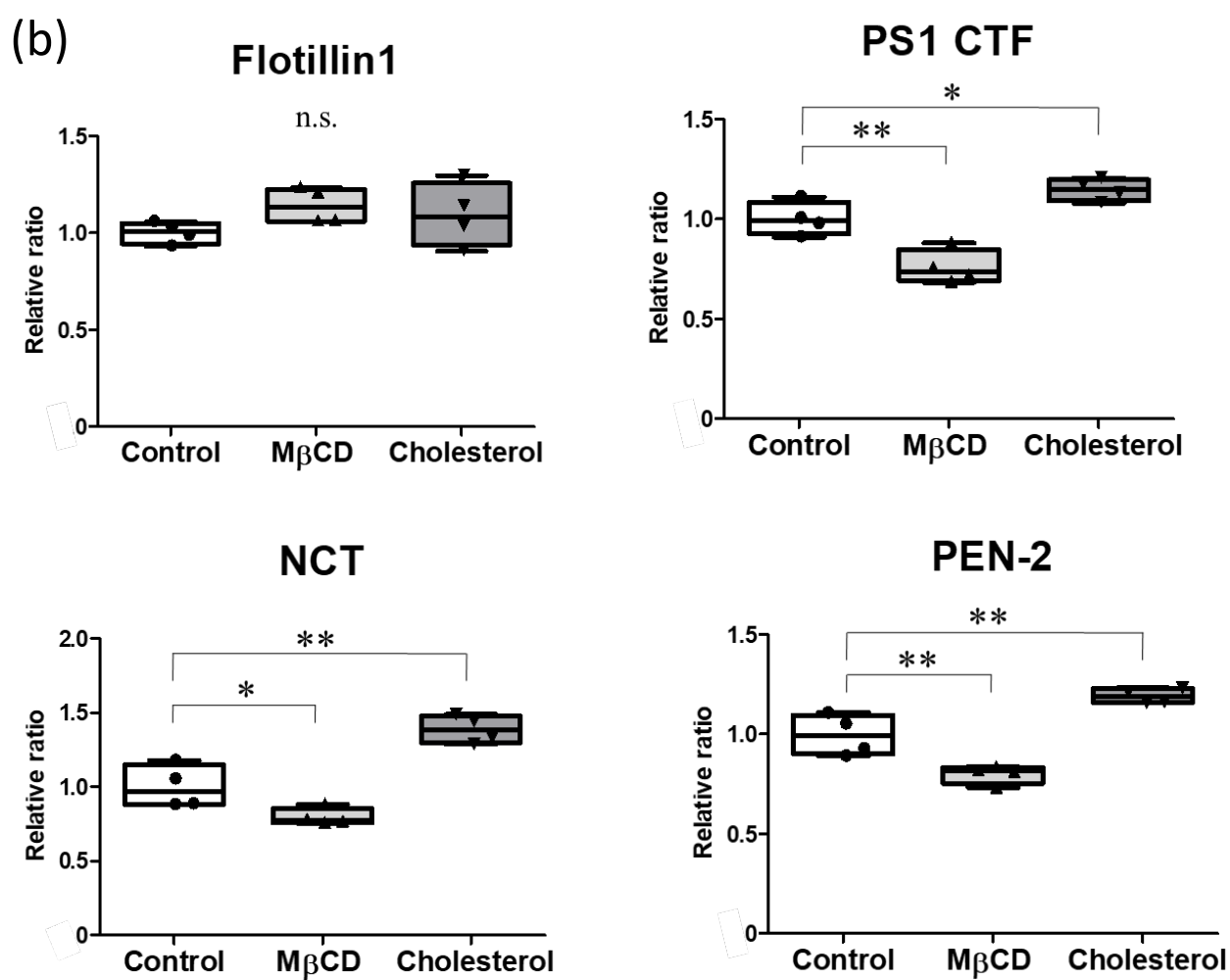
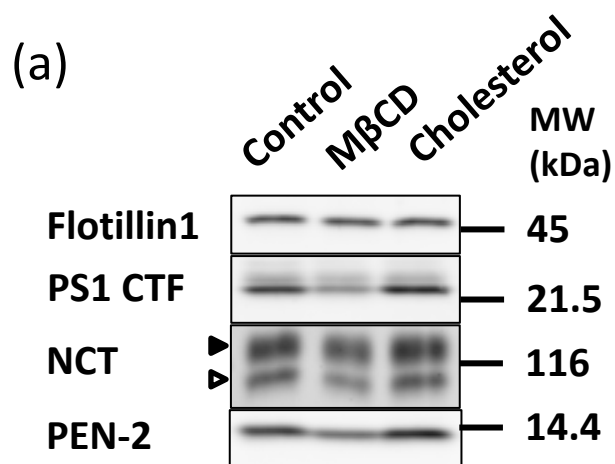
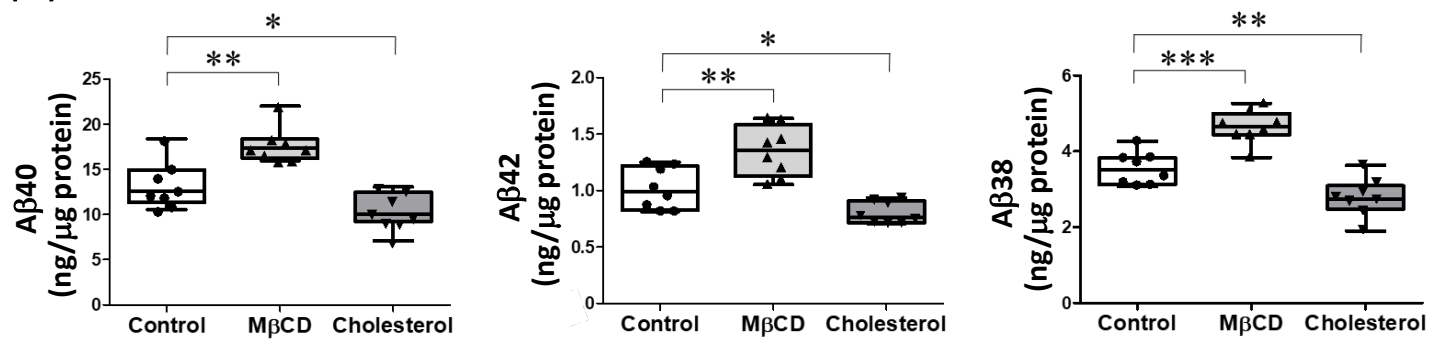


Fig. 5

(a)



(b)

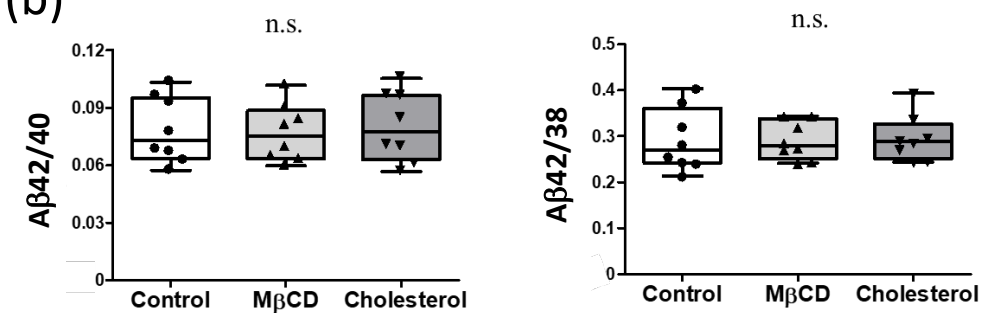
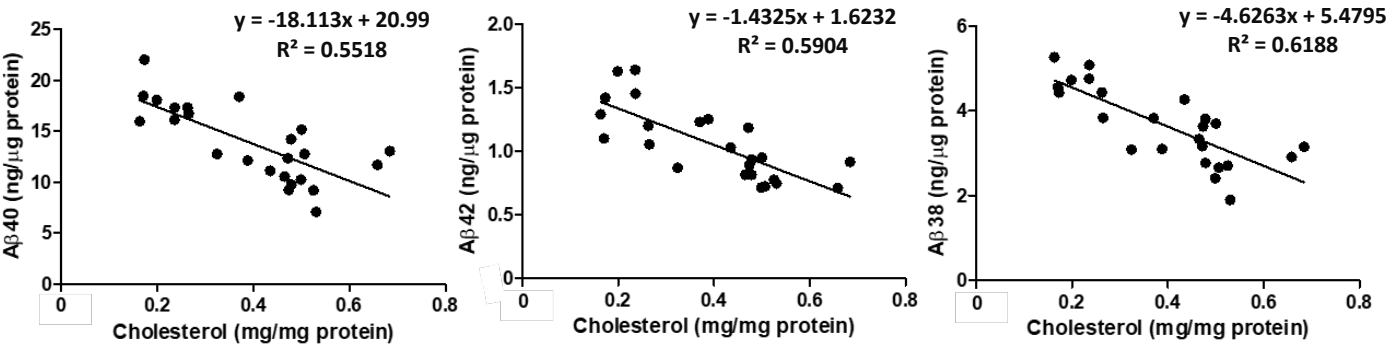


Fig. 6

(a)



(b)

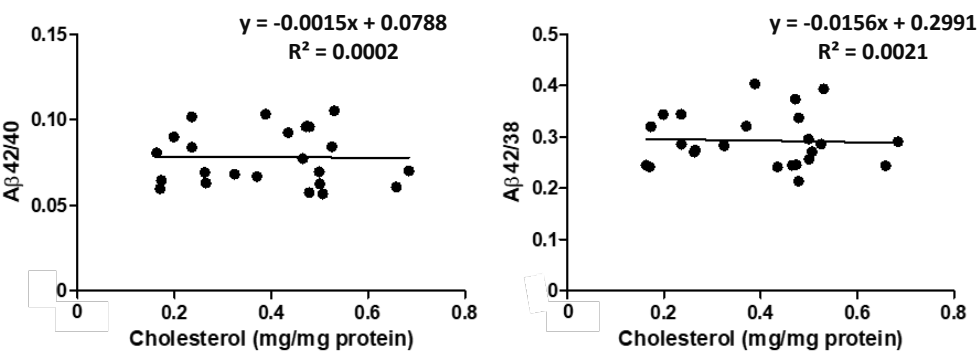
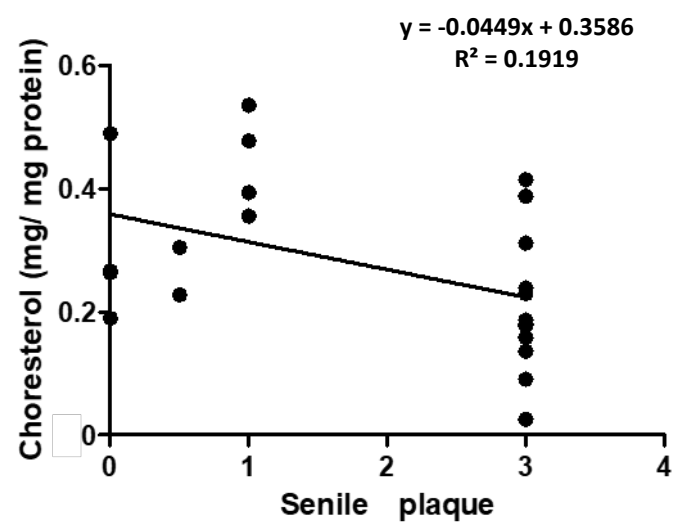


Fig. 7

(a)



(b)

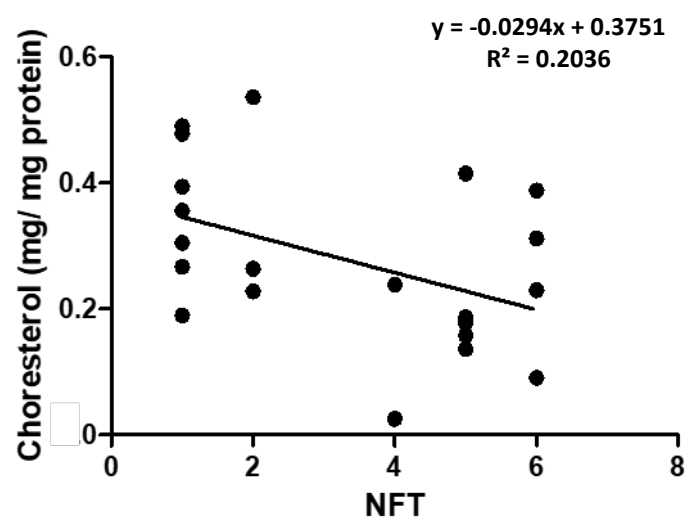
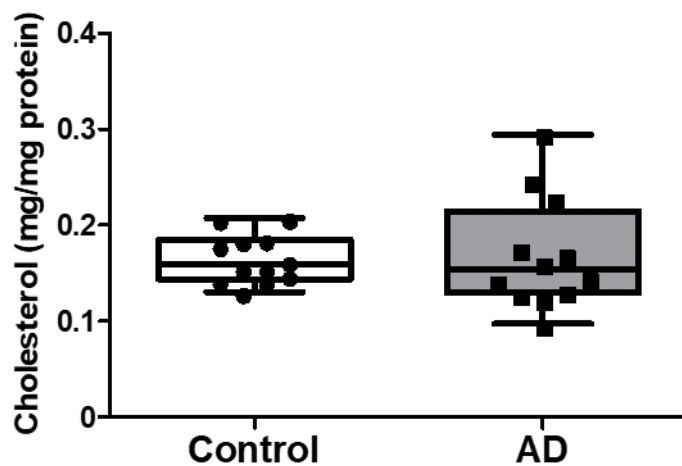


Fig. 8

Supplementary material

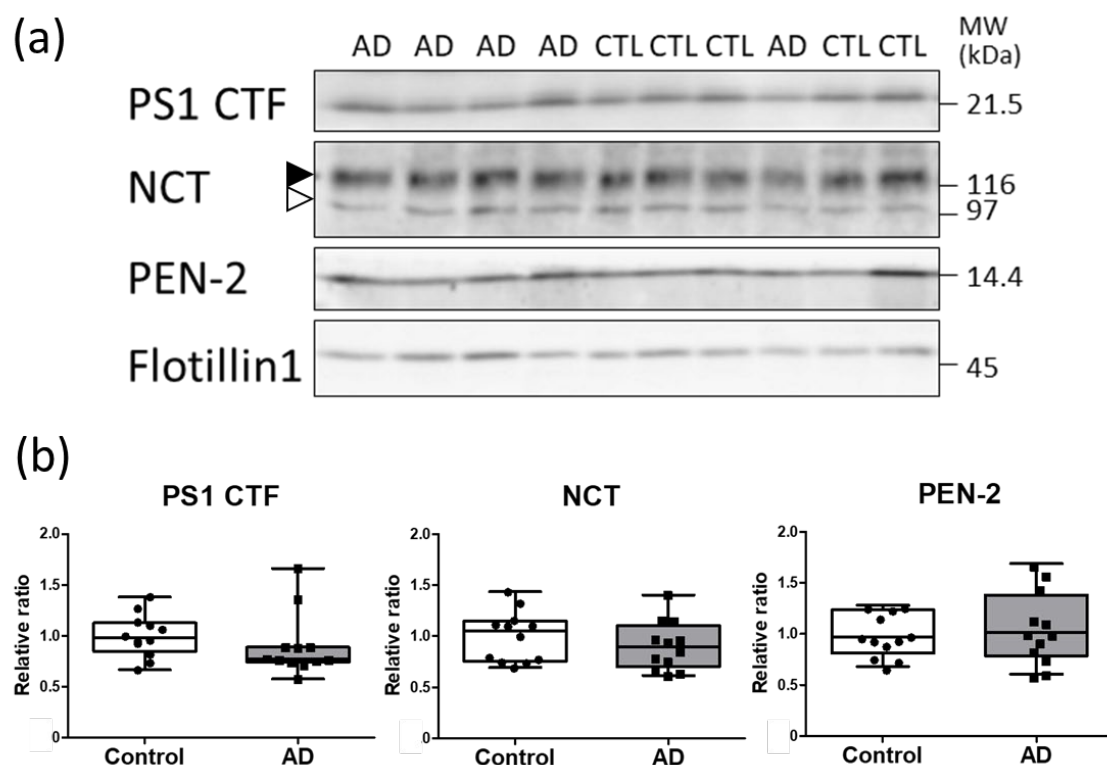
Enhanced amyloid- β generation by γ -secretase complex in detergent-resistant membrane microdomains with reduced cholesterol levels

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Supplementary Fig. S1. Cholesterol contents in total membrane fraction of human brain.

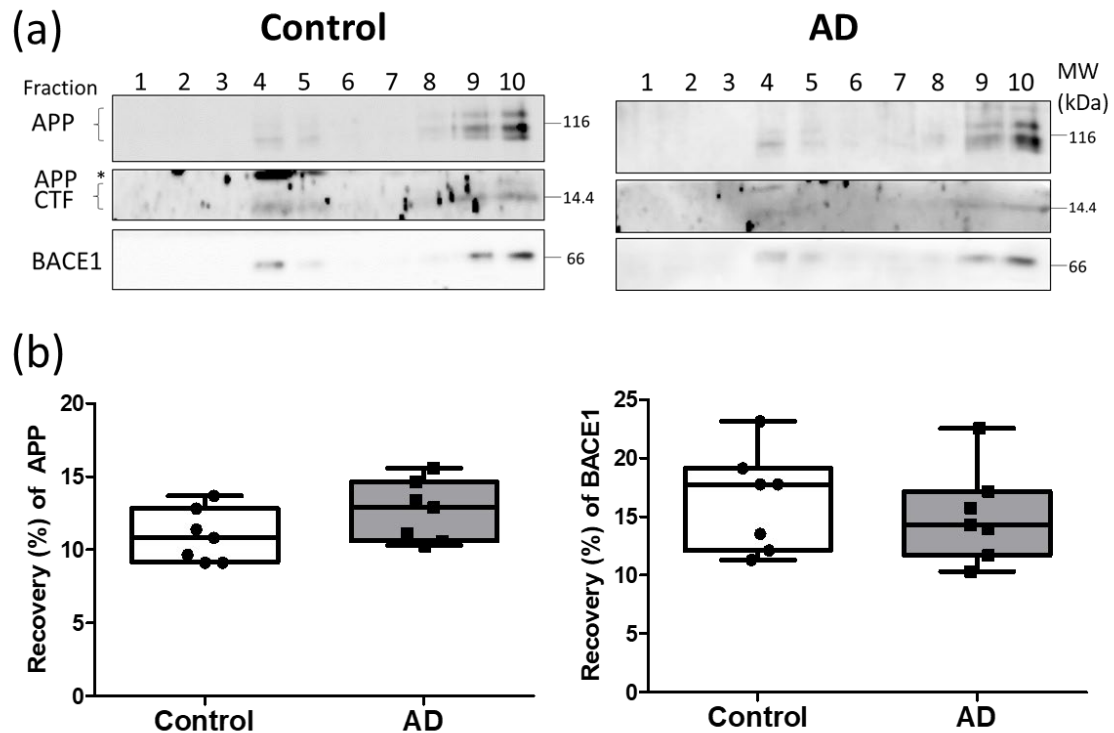
Cholesterol levels of P100 membrane fraction were examined and standardized with protein contents. Data are shown as a box plot. No significance was observed with a statistical analysis of Student's *t* test ($n=12$ AD, 10 control).



Supplementary Fig. S2. Relative amounts of γ -secretase components in total membrane fraction of human brain.

(a) Immunoblot analysis of PS1 CTF, NCT and PEN-2 along with flotillin1. P100 membrane fraction (5 μ g protein) of human brain tissues from nondemented age-matched control (CTL) and AD subjects were analyzed by immunoblotting with PS1 CTF, NCT, PEN-2 and flotillin1 antibodies. In NCT, mature ($\sim$ 120 kDa, closed arrowhead) and immature ($\sim$ 100 kDa, open arrowhead) proteins are detected. Representative blots are shown. The numbers indicate molecular size (kDa).

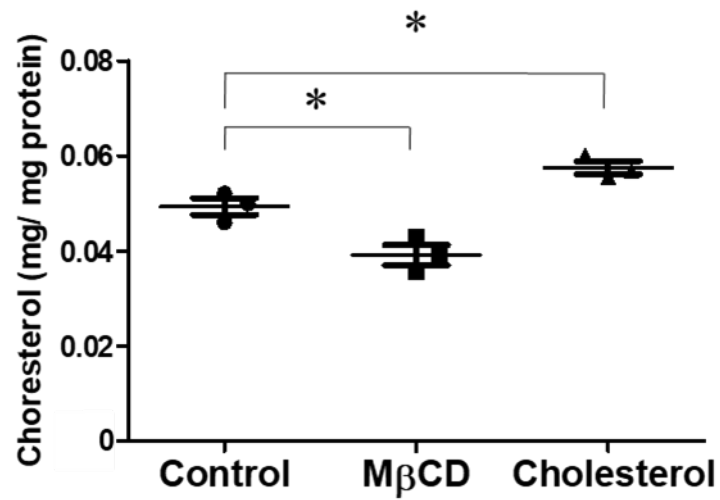
(b) Relative amounts of PS1 CTF, NCT and PEN-2. Respective proteins were quantified with LAS-4000 mini imager (FUJIFILM, Tokyo, Japan) and standardized with flotillin 1. The standardized band densities of AD were compared with those of control (a reference value of 1.0) as a relative ratio. Data are shown as box plots. No significance (PS1 CTF, $P=0.3406$; NCT, $P=0.3669$; PEN-2, $P=0.5668$) was observed with a statistical analysis of Student's t test ($n=12$ AD, 10 control).



Supplementary Fig. S3. Distribution of APP, APP CTF and BACE1 in human brain tissue membranes.

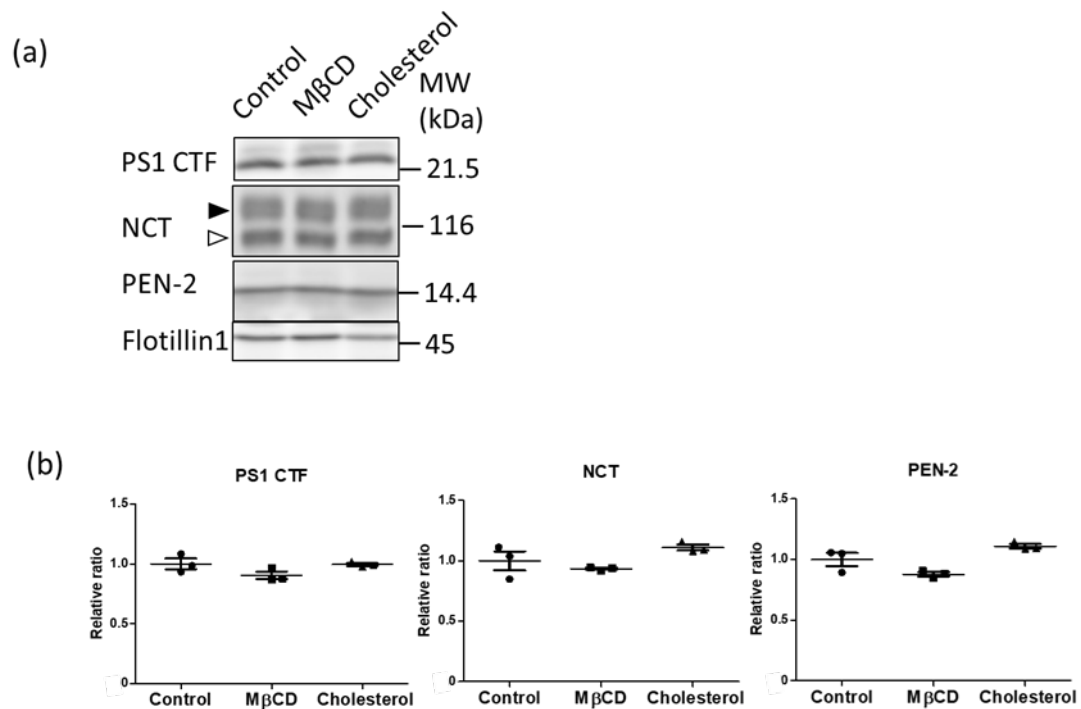
(a) Isolation of DRM and non-DRM fractions and distribution of APP, APP CTF and BACE1. DRM (fractions 4 and 5) and non-DRM (fractions 9 and 10) were isolated as described in the legend of figure 1. Respective fraction samples (24 μ l/lane) were analyzed by immunoblotting to determine the localization of APP, APP CTF (with anti-APP cytoplasmic region antibody) and BACE1 (with anti-human BACE1 antibody). Representative blots are shown. The numbers indicate molecular size (kDa). The asterisk indicates a non-specific band.

(b) Recovery of APP (left) and BACE1 (right) from DRM fractions from nondemented age-matched control (Control) and AD subjects. Recovery was quantified from band densities (panel a), and the recovery from DRM fraction is indicated. Data are shown as box plots. No significance (APP, $P=0.1274$; BACE1, $P=0.5701$) was observed with a statistical analysis of Student's t test ($n=7$ AD, 7 control).



Supplementary Fig. S4. Cholesterol contents in total membrane fraction of HEK293 cells.

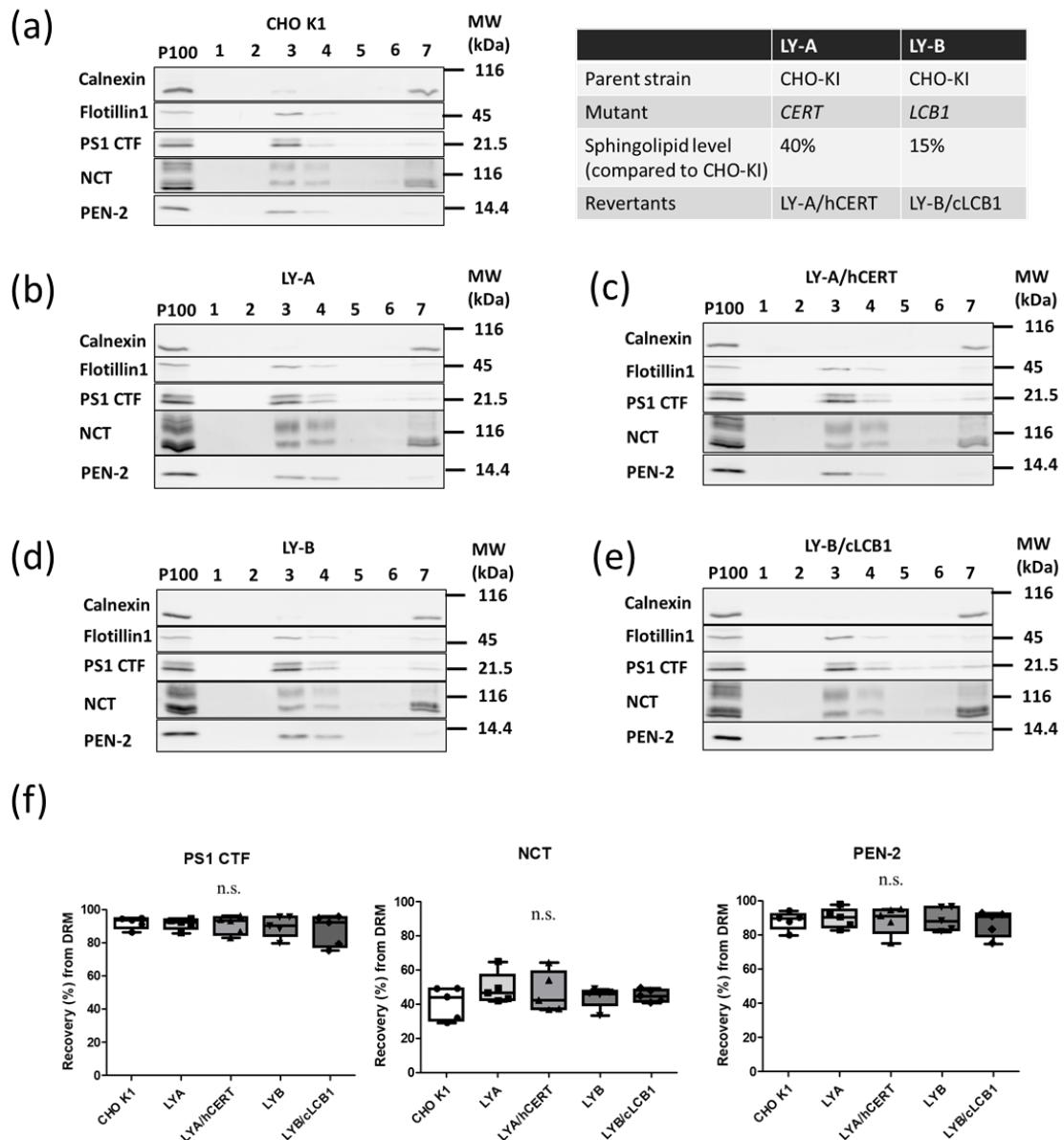
Cholesterol levels of P100 membrane fraction. Cells were treated with MβCD (10 mM), water-soluble cholesterol (0.1 mM), or without reagents (control). Cholesterol contents were standardized with protein amounts. Data represent means $\pm$ SEs. Statistical analysis was performed using Dunnett's multiple comparison test ($n=3$, $*P<0.05$).



Supplementary Fig. S5. Amounts of γ -secretase components in total membrane fraction of HEK293 cells.

(a) Immunoblot analysis of PS1 CTF, NCT and PEN2 along with flotillin1. Equal amounts (3.2 μ l/lane) of P100 membrane fractions prepared from HEK293 cells treated with M β CD (10 mM), water-soluble cholesterol (0.1 mM), or without reagents (control) were analyzed by immunoblotting with PS1 CTF, NCT, PEN2 and flotillin1 antibodies. In NCT, mature (closed arrowhead) and immature (open arrowhead) proteins are detected. Representative blots are shown. The numbers indicate molecular size (kDa).

(b) Relative Amounts of PS1 CTF, NCT and PEN-2 of P100 membrane fractions of cells treated with or without reagents in panel a. Respective proteins were quantified with LAS-4000 mini imager (FUJIFILM, Tokyo, Japan) and the amount was standardized with flotillin 1. The standardized band densities of treated cells were compared with those of control (a reference value of 1.0) as a relative ratio. Data represents the means $\pm$ SEs. No significance ($P > 0.05$) was observed with a statistical analysis of Dunnett's multiple comparison test ($n=3$).

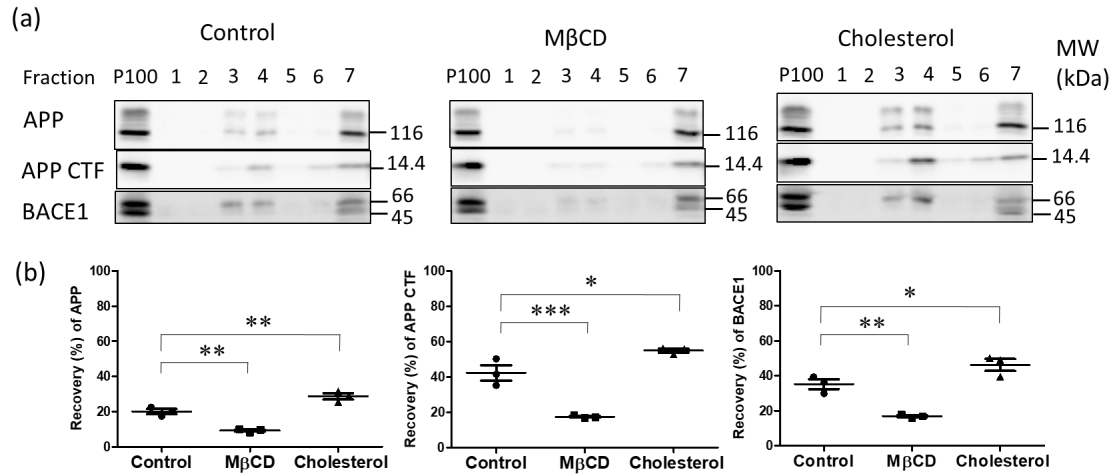


Supplementary Fig. S6. Cells deficient in sphingolipids don't alter distribution of γ -secretase components in DRM fraction.

(a-e) Characterization of γ -secretase components in DRM and non-DRM fractions. DRM (3 and 4) and non-DRM (7) fractions were prepared from P100 membrane fraction of parental wild-type CHO K1 cells (a), LY-A cells (b), LY-A/hCERT, the revertant of LY-A exogenously expressed with human ceramide transfer protein (c), LY-B cells (d), and LY-B/cLCB1, the revertant of LY-B exogenously expressed with yeast serine palmitoyltransferase (e). Membrane fractions (10 μ l) were analyzed by immunoblotting to determine the presence of PS1 CTF, NCT, and PEN-2 along with calnexin (non-DRM resident) and flotillin 1 (DRM resident). Representative blots are shown. The numbers indicate

molecular size (kDa).

(f) Recovery of flotillin 1, PS1 CTF, NCT, and PEN-2 from DRM fractions of cells represented in panels (a-e). Proteins were quantitated from bands densities on blots shown in panels a-e, and the recoveries in DRM fractions (3 and 4) are indicated. Statistical analysis was performed using Dunnett's multiple comparison test ($n = 5$) and no significance was detected. Data are shown as box plots. n.s., not significant.



Supplementary Fig. S7. Distribution of APP, APP CTF and BACE1 in membrane of HEK293 cells.

(a) Isolation of DRM and non-DRM fractions and distribution of APP, APP CTF and BACE1. DRM (fraction 4) and non-DRM (fraction 7) were isolated from cells treated with M β CD (10 mM), water-soluble cholesterol (0.1 mM), or without reagents (control) as described in the legend of figure 3.

Respective fraction samples (10 μ l/lane) were analyzed by immunoblotting to determine the localization of APP, APP CTF (with anti-APP cytoplasmic region antibody) and BACE1 (with anti-human BACE1 antibody). Representative blots are shown. The numbers indicate molecular size (kDa).

(b) Recovery of APP (left), APP-CTF (middle) and BACE1 (right) from DRM fractions from cells treated with or without reagents. Recovery was quantified from band densities (panel a), and the recovery from DRM fraction is indicated. Data represents the means $\pm$ SEs. Statistical analysis was performed using of Dunnett's multiple comparison test ($n=3$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$).