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博士学位論文

Mechanism of altered cleavage of substrates by γ -secretase including presenilin without pathogenic gene mutations

(遺伝子変異に依存しない γ セクレターゼによ
る基質の切断変化機構の解明)

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Abbreviations

AA: amyloid angiopathy
A β : amyloid- β protein
AD: Alzheimer's disease
AICD: APP intracellular domain
Alc: Alcadin (Alzheimer's disease-related cadherin-like protein)
APH-1: anteriorpharynx-defect-1
ApoE: apolipoprotein E
APP: amyloid- β precursor protein
CDR: clinical dementia rating
CHAPSO: 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propane sulfonate
CSF: cerebrospinal fluid
CTF: carboxy-terminal fragment
CVD: cerebral vascular disease;
DAPT: *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester
DMEM: Dulbecco modified Eagle medium
DRM: detergent-resistant membrane fraction
ECL: enhanced chemiluminescence
EDTA: ethylene diamine *N*, *N*, *N*, *N*-tetraacetic acid
EGTA: ethylene glycol tetraacetic acid
FAD: familial Alzheimer's disease
FBS: fetal bovine serum
HBS-T: HEPES buffered saline with Triton X-100
HEK293: human embryonic kidney cells 293
HEPES: 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid
IP: immunoprecipitation
kDa: kilo Dalton
M β CD: methyl- β -cyclodextrin
MCI: mild cognitive impairment
NCT: nicastrin
NFT: neurofibrillary tangles
NTE: amino-terminal fragment
p3-Alc: p3-like peptide generated by serial primary and secondary cleavages of Alc

PBS: phosphate-buffered saline
PEN-2: presenilin enhancer2
PIPES: piperazine-1,4-bis (2-ethanesulfonic acid)
PS/PSEN: presenilin
PSP: progressive supranuclear palsy
SAD: sporadic Alzheimer's disease
sAPP: secreted large amino-terminal ectodomain fragment of APP
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sELISA: sandwich enzyme-linked immunosorbent assay
TBS-T: Tris-buffered saline with tween 20
Tris: tris (hydroxymethyl) aminomethane

Introduction

Alzheimer's disease (AD)

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, which is the most common form of dementia. Like other types of dementia, Alzheimer's is caused by brain cell death (Selkoe *et al.*, 1991). The incidence of AD is rising in line with the aging population. AD can be divided into two types, familial AD (FAD) and sporadic AD (SAD), based on age of onset and genetic predisposition. FAD is also known as early-onset AD, usually strikes earlier in life and is diagnosed before the age of 65. FAD is an uncommon form of AD, caused by a mutation in one of 3 associated genes, accounting for only 5% of all AD cases. In contrast to FAD, SAD is known as late-onset AD, begins after the age of 65 years. SAD is the most common form of AD affecting about 95% of AD patients. However, the molecular and cellular mechanisms remain unknown for SAD without mutations in AD causative genes. Currently, the *APOE* (apolipoprotein E) gene is the only gene identified that is related to SAD (Corder *et al.*, 1993), and aging, midlife hypertension, hypercholesterolemia, diabetes, and obesity can significantly contribute to the development and progression of AD (Kern *et al.*, 2006; Martin *et al.*, 2006). Although FAD and SAD have different age and cause of onset, they do not differ in clinical features. The common pathological features of FAD and SAD patients are the accumulation of senile plaques, and neurofibrillary tangles (NFT) in the brain. As for the possible mechanism, the most widely-accepted amyloid cascade hypothesis suggests that abnormal Amyloid- β (A β) precursor protein metabolism results in A β aggregation and form senile plaques. This leads to the formation of intracellular NFT, primarily composed of the protein tau, and eventually loss of synapses and neurons (Hardy *et al.*, 1991; Haass *et al.*, 2001; Hardy *et al.*, 2002). Therefore, understanding the processing and metabolism of APP is crucial for AD therapeutics.

Amyloid precursor proteins (APP) processing

APP is a type I transmembrane protein and composed of a long N-terminal domain and a short cytoplasmic domain. There are three major splicing isoforms of APP, APP695, APP751, and APP770. Of the three, APP751 and APP770 are expressed in most tissues while APP695 is the major neuronal splice variant (Rohan *et al.*, 1997; Kang *et al.*, 1990).

APP undergoes different proteolytic processing by different sets of enzymes. About 90% of APP is processed through the non-amyloidogenic pathway, and 10% the amyloidogenic pathway which leads to amyloid plaque formation (**Figure 1**). In the non-amyloidogenic pathway, APP is firstly cleaved by α -secretase to generate an extracellular N-terminal fragment sAPP α . Then the remaining C-terminal fragment α (CTF α) is cleaved by γ -secretase to release p3, which is metabolically labile

and cannot form senile plaque. In the amyloidogenic pathway, APP is cleaved first by β -secretase and generate sAPP β and a membrane-associated domain APP CTF β . The APP CTF β is further cleaved by γ -secretase to generate APP intercellular domain (AICD) inside the cell as before, and release A β into extracellular spaces (Kamal *et al.*, 2001). Aggregation of neurotoxic A β then deposits and forms the senile plaque. The promotion of amyloidogenic pathway by some reasons which increase the neurotoxic A β production, is considered as the pathogenesis of AD.

A β production

The A β production has an important connection with γ -secretase activity. γ -Secretase firstly cleaves APP CTF β at ϵ -site to release AICD, then cleaves at several γ -site to generate different kinds of A β products. The most common forms of A β are A β 40 and A β 42, in which A β 40 is the predominant species while A β 42 has higher tendency to form neurotoxic aggregates (Jarret *et al.*, 1993; Iwatsubo *et al.*, 1994). It is reported that A β has two production lines (**Figure 2**). The line converts A β 49 to A β 43 then into A β 40 is the major line, and the line converts A β 48 to A β 42 then into A β 38 is the minor line (Kakuda *et al.*, 2012; Okochi *et al.*, 2013). The neurotoxicity of shorter form of A β , A β 40 or A β 38 is very weak or non-neurotoxic, while the longer forms of A β are highly neurotoxic and prone to aggregation.

Missense mutations that are causative of FAD have been identified in three genes that are essential for the generation of A β peptides: the *APP* gene and two homologous genes that encode the catalytic subunit of γ -secretase, *PSEN1* (encoding presenilin-1) and *PSEN2* (encoding presenilin-2) (Bertram & Tanzi, 2008; Selkoe *et al.*, 2001). Both *APP* and *PSEN* mutations drive amyloidosis in FAD patients through changes in the brain metabolism of A β peptides that promote the formation of pathogenic aggregates. However, in SAD which the *APP* or *PSEN* gene is not mutated, the mechanism of accumulated A β is still unclear. Researches on SAD propose that the progressive accumulation of A β in specific areas of the brain of patient with SAD starts as a result of an imbalance between the A β production and clearance (Hardy & Selkoe, 2002; Mawuenyega *et al.*, 2010).

Some SAD may involve γ -secretase dysfunction

γ -Secretase is a multi-subunit protease complex, which is composed of presenilin enhancer 2 (PEN-2), presenilin (PS), nicastrin (NCT) and anteriorpharyns-defect-1 (APH-1) (**Figure 3**). Presenilin is the catalytic subunit of the γ -secretase complex, and APH-1 stabilizes the presenilin in the complex, whereas PEN-2 is required for endoproteolytic processing of presenilin and conferring γ -secretase activity to the complex (Takasugi *et al.*, 2003). Nicastrin is reported to function as a γ -secretase-substrate receptor (Shah *et al.*, 2005). There are many substrates reported to be cleaved by γ -secretase, such as APP, Alcadin, Notch, ErbB-4, E-cadherin, CD44, APLP1, APLP2, LRP2, Nectin1- α , Delta, Jagged and many other type I membrane proteins (De strooper *et al.*, 2003; Hata *et al.*, 2009).

Previous research quantified A β 40 and A β 42 in cerebrospinal fluid (CSF) samples from control subjects, mild cognitive impairment (MCI, prodromal of AD) and AD patients. The CSF concentrations of A β 42 were found to be significantly lower in MCI/AD compared with controls. This decrease in CSF A β 42 concentration may be due to the fact that, as A β 42 aggregates into fibrils and plaques in the brain, a lower amount of A β 42 is able to diffuse into the CSF. The ratio of A β 42/40 was also generally lower in MCI/AD patients compared to control subjects. These results suggest that the activity of brain γ -secretase in MCI/AD is changed (Kakuda *et al.*, 2012).

Another research of our lab also showed evidence that SAD may involve γ -secretase dysfunction. Alcadin (Alc) is a type I membrane protein which plays important roles in vesicular transport at the subcellular level and in learning behavior at the organismal level (Araki *et al.*, 2007; Ikeda *et al.*, 2009). Alc and APP were colocalized in healthy mouse and AD human brain, particularly in dystrophic neurites in senile plaques, which means Alc may be related to the A β production and development of AD (Araki *et al.*, 2003). The proteolytic processing of Alc proteins appears highly similar to that of APP (**Figure 4A**). Alc is first cleaved by α -secretases. Then the membrane-associated C-terminal fragment (Alc CTF) is cleaved by γ -secretase to release a soluble p3-Alc and a membrane-bound C-terminal fragment AlcICD (Araki *et al.*, 2004; Hata *et al.*, 2009). Same as APP, cleavages of Alc by γ -secretase also have major and minor production lines. Alc has three isoforms, Alc α , Alc β , and Alc γ . Previous research showed that, in Alc α , the p3-Alc α 35 is the major γ -secretase cleavage product as is the A β 40 in APP, and p3-Alc α 38 is the minor product as is the A β 42 in APP (**Figure 4B**). Unlike APP, minor product of Alc α , p3-Alc α 38, is not easy to aggregate. Previous research showed that the ratio of p3-Alc α 38/total p3-Alc α in CSF was elevated in MCI subjects (Hata *et al.*, 2011), and the concentration of p3-Alc α 38 in CSF was elevated in some of the SAD subjects (unpublished data of our lab).

These results suggest that some SAD may involve alternative processing of multiple γ -secretase substrates, raising the possibility that the molecular pathogenesis of SAD might involve γ -secretase dysfunction.

The purpose of this research

The mechanisms underlying the altered cleavages of multiple substrates by γ -secretase in SAD remain uncovered.

It has been proposed that γ -secretase mainly localizes in cell membrane microdomains called lipid rafts, and cleavages of substrates by γ -secretase also occur in this special region (Vetrivel *et al.*, 2010; Urano *et al.*, 2005). Lipid rafts are characterized by their high content in cholesterol and sphingolipids (**Figure 5**), play an important role in many cellular processes including signal transduction, membrane trafficking, cytoskeletal organization, and pathogen entry (Pike *et al.*, 2003; Munro S, 2003). Cholesterol is a critical structural component of lipid rafts, and depletion of

cholesterol leads to disorganization of lipid rafts and dissociation of proteins bound to the lipid rafts (Hanada *et al.*, 1995). However, the role of cholesterol in AD is highly controversial. For example, some studies show that the plasma cholesterol level or the brain cholesterol level was elevated in AD patients compared with controls, while other studies show no differences or opposite results (Wood *et al.*, 2014). Thus, the role of cholesterol in AD remains to be determined. Experimental variation of the proportions of various membrane lipids has been demonstrated to alter the C-terminal speciation of A β (Holmes *et al.*, 2012), and the cholesterol level in lipid raft was reduced in AD brains (Molander-Melin *et al.*, 2005). Thus, we hypothesized that altered cholesterol level in lipid rafts of membrane may influence γ -secretase cleavages of substrates. To confirm our hypothesis, here we explored the γ -secretase ability to produce A β *in vitro* with altered cholesterol level in lipid rafts of cells or brain neuron membranes.

Results

1. Analyzing the cholesterol level and γ -secretase activity in lipid rafts of AD patients

Active γ -secretase complex comprising of the endoproteolytically processed presenilin 1 or 2 amino-terminal fragment (PS1 NTF or PS2 NTF) and carboxyterminal fragment (PS1 CTF or PS2 CTF), NCT, APH-1 and PEN-2 largely localizes in a membrane lipid raft-like microdomain, which can be isolated biochemically as a detergent CHAPSO-resistant membrane (DRM) (**Figure 6A**) (Saito *et al.*, 2008; Sawamura *et al.*, 2000).

We prepared DRM from brain samples of SAD and age-matched non-demented subjects (subjects' information is shown in **Table 1**). Flotillin1, the resident protein in lipid raft, is regarded as the marker of DRM fraction while calnexin is used as the marker of non-DRM fraction. The fractions (fractions 4 and 5) which flotillin1 is largely localized were used as DRM fraction (**Figure 6B**). We also analyzed distribution of PS1 CTF. PS1 CTF was largely recovered only from DRM fractions from control samples but was present in non-DRM fractions from AD samples (**Figure 6B**). Moreover, the recovery of PS1 CTF from DRM fractions of AD subjects was significantly lower than from control subjects (**Figure 6C**), 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* ϵ 4 carriers (indicated with open symbols in **Figure 6C**).

Previous report shows that the cholesterol level in DRM was lowered in AD brains compared to non-AD controls (Molander-Melin *et al.*, 2005), therefore, we examined the cholesterol levels in DRM samples of AD and non-demented subjects. We found that the DRM cholesterol level of SAD subjects significantly lower than the non-demented controls (**Figure 6D**).

We next asked whether DRM fractions from AD subjects had an altered ability to generate A β when compared to DRM from controls. DRM from AD and non-demented controls were examined for A β generation with the C99/CTF β -FLAG substrate by *in vitro* γ -secretase assay (**Figure 7A**). Generated A β s were quantified by sandwich enzyme-linked immunosorbent assays (sELISA). Both A β 40 and A β 42 generation significantly elevated in DRM from AD relative to non-demented controls (**Figure 7B**). Ratio of A β 42/A β 40 was also higher in AD than in non-demented controls (**Figure 7C**), as observed in FAD subjects harboring mutation in *PSEN* genes (Schuner *et al.*, 1996; Olsson *et al.*, 2014; Takami *et al.*, 2009), indicating that AD subjects who show an aberrant γ -secretase membrane distribution have a tendency to produce more amounts of A β and neurotoxic A β species, despite the absence of FAD mutations in *PSEN* genes. Interestingly, DRM from *APOE* ϵ 4 carriers (open symbol) showed typically lower cholesterol levels and higher ability to generate A β 40 and A β 42 among AD subjects, suggesting that *APOE* ϵ 4 may contribute to decrease the cholesterol level of brain membrane

DRM.

Taken together, human brain analysis suggests a hypothesis that the lowered cholesterol contents in brain DRM microdomain from AD subjects alters the membrane distribution of γ -secretase components and enhances overall production of A β .

2. Influence of cholesterol level on γ -secretase activity in DRM from cells

High quality human brain resource is precious and is not suitable for further biochemical analysis, and may be various as a bioresource. 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 remove cholesterol from cells, or water-soluble cholesterol (cholesterol-saturated M β CD) to supply cholesterol contents. DRM fractions were prepared from these cells. Treatment of cells with M β CD decreased the cholesterol level in DRM by ~50% relative to the DRM from non-treated control cells, whereas the treatment of cells with cholesterol-saturated M β CD increased the level significantly (**Figure 8C**). Membrane distribution of γ -secretase components was analyzed and the recovery of PS1 CTF, NCT and PEN-2 into DRM was examined by immunoblotting (**Figure 8A**). A β -1 was not examined because we could not obtain the antibody. The recoveries of respective γ -secretase component in DRM (fractions 4 and 5 containing flotillin-1) and non-DRM (fraction 7 containing calnexin) were determined. As expectedly, the γ -secretase components PS1 CTF, NCT and PEN-2 dispersed from DRM into non-DRM from cells treated with M β CD. 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 (**Figure 8B**). These results were similar to those for AD tissue samples. By contrast, the recovery of these γ -secretase components was significantly higher in cells treated with cholesterol-saturated M β CD than in controls, suggesting that the amount of cholesterol in the membrane influences the distribution of the γ -secretase complex.

We next examined DRM ability to generate A β using *in vitro* γ -secretase assay with the C99/CTF β -FLAG substrate. The levels of γ -secretase components in the DRM fractions used in the assay were first assessed by immunoblotting because amounts of γ -secretase components may influence the generation of A β (**Figure 9A**). 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 (**Figure 9B**). Lesser amounts of γ -secretase components, PS1 CTF, NCT and PEN-2, were detected in DRM from cells treated with M β CD, while more these components were detected in these treated with cholesterol saturated M β CD compared to DRM from non-treated cells. Using these DRM samples, A β generation was assayed. Interestingly, DRM from cells treated with M β CD increased all A β 40, A β 42 and A β 38 production significantly regardless of the decreased γ -secretase components (**Figure 10A**). Compared to this, DRM from cells treated with cholesterol-saturated

M β CD significantly decreased the generation of all A β species regardless of the increased γ -secretase components. Ratios of A β 42/40 and A β 42/38 were not changed among respective DRM fraction (**Figure 10B**). The study with DRM from cultured cells indicates that the decrease of cholesterol in DRM enhances overall production of A β , which coincides with the result with DRM from human brain samples, although the study using cultured cells could not induce the increase of A β 42/40 ratio which observed in human brain samples (**Figure 7C**). Qualitative changes of A β generation may be due to another factor except for cholesterol level in brain DRM.

3. Possible reasons causing the alteration of cholesterol in DRM from brain

To explore the possible reason causing the reduction of cholesterol level and changes of γ -secretase activity in DRM from SAD patients' brain, we examined the influence of some risk factors using mice.

3-1. Influence of aging on cholesterol level and γ -secretase activity in DRM

Firstly, since aging is the greatest risk factor of AD, we examined the influence of aging on mice. 3-Month-old mice were used as young mouse group, and 2-year-old mice were used as aged group. We prepared DRM from brain cortex of these mice, and the fraction (fraction 5) which flotillin1 is largely localized was used as DRM fraction (**Figure 11A**). We analyzed membrane distribution of γ -secretase components PS1 CTF, NCT and PEN-2 in DRM but we didn't observe altered distribution of γ -secretase components as observed in SAD brain or cell experiment (**Figure 11B**). Then we detected cholesterol level in DRM fraction, but we didn't observe significant changes of cholesterol level in DRM fraction between young and aged groups (**Figure 11C**). We also examined DRM ability to generate A β using *in vitro* γ -secretase assay with the C99/CTF β -FLAG substrate. The production of A β 40, A β 42 and ratio of A β 42/40 did not change significantly (**Figure 12A, B**). These data indicate that aging cannot easily influence the cholesterol level in DRM from neurons within mouse brain, and the production of A β s and membrane distribution of γ -secretase components may not change without the alteration of cholesterol level.

3-2. Influence of diet on cholesterol level and γ -secretase activity in DRM

Diet is also a key factor in the development of AD (Grant, 1999). We next examined the influence of low-fat diet or high-fat diet to alter the cholesterol level and γ -secretase activity in DRM from brain since different fat diet may be directly related to the change of cholesterol level *in vivo*. We fed 4-month-old mice with control diet, low-fat diet or high-fat diet for 7 months. The control diet has 10% fat and 48 mg/kg cholesterol while the low-fat diet has only 5% fat and no cholesterol. The high-fat diet has 35% fat and 301 mg/kg cholesterol (**Figure 13A**).

We recorded the weight of mouse for 7 months (**Figure 13B**). Compare to the control diet, the

weight of mice fed with high-fat diet was increased while the weight of the mice fed with low-fat diet was decreased.

After feeding mice for 7 months, the cortex of mouse brain was used for preparing the DRM fraction. The fraction (fraction 5) which flotillin1 is largely localized was used as DRM fraction (**Figure 14A**). We analyzed membrane distribution of γ -secretase components PS1 CTF, NCT and PEN-2 in DRM but we didn't observe altered distribution of γ -secretase components as observed in SAD brain or cell experiment (**Figure 14B**). Then we detected cholesterol level in DRM fraction, however, we didn't observe significant changes of cholesterol level in DRM fraction among different diet groups (**Figure 14C**). We also examined DRM ability to generate A β using *in vitro* γ -secretase assay with the C99/CTF β -FLAG substrate. The production of A β 40, A β 42 and ratio of A β 42/40 did not change significantly (**Figure 15A, B**). These data indicate that low-fat diet and high-fat diet cannot easily influence the cholesterol level in DRM from neurons within mouse brain.

3-3. Influence of cholesterol level on γ -secretase activity in DRM from mouse brain neurons

According to the above data, aging and diet cannot easily influence the cholesterol level in DRM from mouse brain neurons, and we noticed that the production of A β s and membrane distribution of γ -secretase components did not change without the alteration of cholesterol level.

To further confirm the influence of cholesterol level on the γ -secretase activity in DRM, we changed the cholesterol level of membrane prepared from mouse neurons by treatment with M β CD or cholesterol-saturated M β CD. After treatment, DRM fractions were prepared from these membranes of mouse cortex. The fraction (fraction 5) which Flotillin1 is largely localized was used as DRM fraction (**Figure 16A**). Treatment of mouse membrane fraction with 10 mM M β CD decreased the cellular cholesterol level in DRM by 75% compared to non-treated control, whereas the treatment of cells with 0.01 mM cholesterol-saturated M β CD increased the level by 150% (**Figure 16C**). Membrane distribution of γ -secretase components was analyzed and the recovery of PS1 CTF, NCT and PEN-2 into DRM was examined by immunoblotting (**Figure 16B**). The alteration of membrane distribution of γ -secretase components was not observed. Maybe the condition we used in the mouse experiment was not enough to detect the possible changes, or these alterations of γ -secretase components only occur in living cells. Finally, we investigated the generation of A β 40, A β 42 and A β 38 in DRM by *in vitro* γ -secretase assay with the C99/CTF β -FLAG substrate. We observed significant increase of A β 42 level in DRM from membrane treated with M β CD, while significant decrease of A β 40 and A β 38 in DRM from membrane treated with cholesterol-saturated M β CD (**Figure 17A**). However, we did not observe significant change of the A β 42/40 or A β 42/38 ratio (**Figure 17B**). Although these data were not exactly the same as cell experiment, these results confirm that A β production is influenced by the cholesterol level in DRM.

Discussion

In this study, we investigated the possible reason causing the aberrant γ -secretase activity and the onset of SAD.

We found that membrane distribution of the active γ -secretase components is altered in AD brain and cells showing a lowered cholesterol level. Cell model study suggests that the dispersion of PS1 CTF from DRM observed in AD brains is caused by an insufficient supplementation of cholesterol into DRM from neurons. There is a slight difference between cell model study and AD brains. DRM from cell model treated with M β CD increased overall production of A β species, while DRM from AD brain increased A β 42/40 ratio along with the increase of both A β production. Nevertheless, we propose that the lower cholesterol in DRM enhances total A β production regardless of the decreased γ -secretase components in DRM and this may be a cause of SAD.

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 A β PH-1) of the large γ -secretase complex, it is believed that these solubilized components cannot form a structure with full enzyme activity. 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.

Exact molecular mechanism how γ -secretase enhances A β generation in DRM with lower cholesterol level remains unclear, however, it is possible to consider that APP CTF can easy access to γ -secretase complex in DRM when cholesterol level lowered or vice versa by an increased membrane fluidity. To be more specific, we guess that reduced cholesterol level decreases the localization of some related protein such as γ -secretase components in DRM, resulting in a relatively loose structure of DRM, while elevated cholesterol increases the localization of some related protein in lipid raft, resulting in a relatively compact structure of DRM. Then we propose two hypotheses of possible mechanisms. Firstly, due to the loose structure in DRM with reduced cholesterol level, the APP CTF can easy access to γ -secretase and be cleaved by γ -secretase leading to more A β generation (**Figure 18A**). On the contrary, the APP CTF become harder to access to the γ -secretase in DRM with elevated cholesterol level due to the compact structure in DRM. Secondly, the loose structure of DRM with reduced cholesterol level increase membrane fluidity, which allows more APP CTF entering DRM followed by γ -secretase cleavage and generate more A β (**Figure 18B**). On the contrary, the compact structure of DRM with elevated cholesterol level decrease membrane fluidity, which allows lower APP CTF entering DRM followed by γ -secretase cleavage and generate lower A β . Although the quantity

of γ -secretase components is decreased in DRM with reduced cholesterol level, the cleavage of APP CTF by γ -secretase is increased due to the above possible mechanism, resulting in increased A β generation.

Moreover, we investigated the possible reason causing the reduction of membrane cholesterol level in SAD. Multiple factors such as aging, life style, gene mutations and environment are reported to be the risk factors of SAD. In this study, we examined two risk factors, aging and diet, by examining cholesterol level and γ -secretase activity in DRM from brain neurons using mouse model. However, our results showed that cholesterol level, membrane distribution of the γ -secretase components or A β generation did not change in DRM from aging mice or mice fed with low-fat or high-fat diet. Aging is the greatest risk factor of SAD which is marked by cognitive impairment and the build-up of amyloid-protein plaques in the brains of human. However, AD does not occur naturally in wide-type mice. In contrast, many non-human primates naturally develop A β plaques due to the highly conserved APP sequence between human and non-human primate APP (Struble *et al.*, 1985; Selkoe *et al.*, 1987). We cannot rule out the effect of aging on the alteration of membrane cholesterol level and γ -secretase activity in DRM from human brain, for the reason that mice might not be a suitable model because they don't naturally get AD due to the great genetic and physiological difference between mice and human. Non-human primates such as monkey might be a better model for this study, however, we did not use monkey because we don't have enough monkey brain samples. We will try to examine the effect of aging on the alteration of membrane cholesterol level and γ -secretase activity in DRM from monkey brain sample in the future. As for the diet, compared to control group, although we observed reduced weight in mice fed with low-fat diet and elevated weight in mice fed with high-fat diet, we did not observed changes in cholesterol level in DRM from brain neurons. These results indicate that diet might not easily influence the cholesterol level in DRM from brain neurons, which may due to the blood-brain barrier prevents brain cells from taking up cholesterol from the blood, and cholesterol is primarily supplied by de novo syntheses in brain (Pitas *et al.*, 1987).

Further experiments are needed to investigate the possible reasons that influence cholesterol level and γ -secretase activity in DRM from brain of other animal models. Apolipoprotein E (ApoE), a protein that transports cholesterol in the central nervous system and influences A β aggregation and clearance in the brain. It exists in three major isoforms: ApoE2, ApoE3 and ApoE4, with frequencies of 7–8%, 77–78% and 14–15% respectively in the general population (Cedazo-Minguez and Cowburn *et al.*, 2001), which result from the expression of the ϵ 2, ϵ 3 and ϵ 4 alleles of the *APOE* genes (Zannis *et al.*, 1981). Although the three common isoforms differ by only one or two amino-acids at residue 112 or 158, these differences profoundly alter ApoE structure and function (Mahley *et al.*, 2006). After immunoreactivity of ApoE in amyloid plaques was first reported (Namba *et al.*, 1991; Wisniewski and Frangione, 1992), the ϵ 4 allele of the *APOE* gene was discovered to be a strong genetic risk factor for AD (Corder *et al.*, 1993; Strittmatter *et al.*, 1993a). People with a single copy of *APOE* ϵ 4 have a 3-

fold greater risk of developing AD; those with two copies have a 12-fold greater risk (Bertram *et al.*, 2009; Roses, 1996). Our experiment of human brain involved some *APOE* $\epsilon 4$ carrier AD subjects. Interestingly we observed that brain DRM from *APOE* $\epsilon 4$ carrier AD subjects contains lower levels of cholesterol among AD subjects and expresses an ability to generate more A β compared to non-AD controls with higher cholesterol in DRM. This result indicates that *APOE* $\epsilon 4$ might be another candidate causing the reduction of cholesterol level and aberrant γ -secretase activity in DRM from SAD subjects' brain. In the future, we will examine this possibility using *APOE* knock-in mice expressing different human ApoE isoforms.

In conclusion, although we cannot rule out other factors that induce the alteration of A β generation in sporadic AD, current results suggest that upregulated production of A β observed in the dispersion of active γ -secretase component from DRM by lowering the cholesterol level in DRM may be a potent cause of sporadic AD pathogenesis.

Material and methods

Cell culture

HEK293 cells were maintained with DMEM (Wako) including 5% (v/v) of the inactivated FBS (BIOSCIENCE) and antibiotics mix (70 µg/ml penicillin (Wako), 100 µg/ml streptomycin (Wako), and 2.5 µg/ml amphotericin B (Wako)) at 37°C in a humidified cell culture chamber under 5 % CO₂. Cells were split when they were 80-90% confluent.

Sf9 cells were maintained with PSFM-J1 medium including 2.5% (v/v) of the inactivated FBS (BIOSCIENCE) and Pen Strep (Gibco, 100 µg/ml penicillin and 100 µg/ml streptomycin) at 27°C in a cell culture chamber. Cells were split when they were 80-90% confluent.

Preparation of C99/CTFβ-FLAG Substrate

APP CTFβ (C99) tagged with FLAG at the carboxyl terminus (C99/CTFβ-FLAG) was prepared from Sf9 cells basically as described (Kakuda *et al.*, 2006). Briefly, Sf9 cells were infected with recombinant baculovirus and cultured in the presence of 20 µM GM6001 to suppress α-secretase-like cleavage of C99/CTFβ-FLAG. C99-FLAG overexpressed in Sf9 cells was solubilized with the lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, and protease inhibitor mixture) and then immunoaffinity-purified with anti-FLAG® M2-agarose beads (Sigma). The quantity and purity of the obtained C99/CTFβ-FLAG were assessed by gel electrophoresis and Coomassie Brilliant Blue staining.

Mouse

All mouse experiments in this study were conducted after obtaining approval from the Institutional Animal Care and Use Committee of the National University Corporation at Hokkaido University (No. 18-0065) in accordance with Hokkaido University Regulations of Animal Experimentation. Mice were bred under SPF conditions and housed in individual plastic cages that were maintained at a constant temperature (26°C) under a 12-h light/dark cycle with water and food provided ad libitum. Control diet (TestDiet® #5755), low-fat diet (TestDiet® #58B0) and high-fat diet (TestDiet® #58Y1) were used in the experiment examining the influence of diet to alter cholesterol level and γ-secretase activity in DRM from brain neurons.

Autopsy human brain tissues

Frozen brain tissues from the frontal and temporal cortices of twelve AD patients and ten non-neurological disease control subjects were obtained from the Brain Bank for Aging Research, Tokyo Metropolitan Institute of Gerontology (Tokyo, Japan); the Medical Science Review Board of Shiga

University of Medical Science approved the study protocol. Ethical boards at Hokkaido University Faculty of Pharmaceutical Sciences approved the study used human samples. Alzheimer's disease was clinically diagnosed based on the criteria of the National Institute of Neurological and Communicational Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA). Subject information is provided in **Table 1**. Staging of “senile plaque” and “NFT” are followed by Braak's criteria (Braak & Braak 1991; Braak 2006)

Depletion/incorporation of cholesterol in cell or mouse brain membrane

Cells:

2x10⁶ HEK293 cells were spread on 10 cm dish. After 72 h incubation, the medium was changed to serum-free DMEM. To deplete cellular cholesterol, the cells were cultured in the presence of 10 mM M β CD (Wako) for 1 h. Alternatively, to incorporate cholesterol, the cells were cultured for 30 min in the presence of 0.1 mM water-soluble cholesterol (Sigma), which consists of M β CD containing cholesterol. After incubation, the cells were washed with PBS (10 mM sodium phosphate buffer pH 7.4, 137 mM NaCl, 27mM KCl) and then harvested for preparing the DRM fraction.

Mouse brain membrane:

Mouse brain cortex was lysed in eight-folds volume of TNE buffer by using Dounce homogenizer (Dounce Tissue Grinder, tight) 30 times followed by passing them through a 27-gauge needle 30 times on ice. The supernatant was further centrifuged at 140,000 x g (48,000 rpm with TLA55; Beckman Coulter, Brea, CA, USA) for 1h. Then the precipitated membrane fraction (P100) was resuspended with TNE, 10 mM M β CD in TNE or 0.1 mM water-soluble cholesterol in TNE. After incubation at 37°C for 30min, the mixture was centrifuged at 48,000 rpm for 1h. The resultant precipitates were resuspended with 1% (w/v) CHAPSO in TNE and subjected to fractionation for DRMs.

Preparation of DRM fraction

Cells harvested from 10 cm dish were lysed in 300 μ l of TNE buffer (10 mM Tris-HCl pH7.5, 150 mM NaCl, 5 mM EDTA) by passing them through a 30-gauge needle 10 times on ice. Unbroken cells and nuclei were removed by centrifugation at 3,000 x g for 10 min at 4°C. The supernatant was further centrifuged at 140,000 x g (48,000 rpm with TLA55; Beckman Coulter, Brea, CA, USA) for 1h, the precipitated membrane fraction (P100) prepared from 6 dishes of cells was resuspended in 500 μ l of TNE and CHAPSO (3-[(3-Cholamidopropyl) dimethylammonio]-2-hydroxypropanesulfonate) (Dojindo, Kumamoto, Japan) was added by 1% (w/v). Detergent (CHAPSO)-resistant membrane (DRM) and non-DRM fractions were prepared by discontinuous sucrose gradient centrifugation. Samples (500 μ l) were mixed with equal volume of 85% sucrose in TNE and then were 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 39,000 rpm (SW41Ti; Beckman Coulter) for 20 h. Fractions (1 ml) were collected from the top (fraction 1) of the ultracentrifuge tubes down to fraction 7.

Human or mouse brain sample were lysed in eight-folds volume of TNE buffer by using Dounce homogenizer (Dounce Tissue Grinder, tight) 30 times followed by passing them through a 27-gauge needle 30 times on ice. The supernatant was further centrifuged at 140,000 x g (48,000 rpm with TLA55; Beckman Coulter, Brea, CA, USA) for 1h, the precipitated membrane fraction (P100) was resuspended in 1 ml of TNE and CHAPSO was added by 1% (w/v). DRM fractions of human or mouse brain 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), which were collected from the top (fraction 1) of the ultracentrifuge tubes down to fraction 10. Proteins in the respective fractions were analyzed by immunoblotting and quantitated with LAS-4000 mini (Fujifilm, Tokyo, Japan). Cholesterol contents of DRM were quantitated using the LabAssay Cholesterol kit (Wako Pure Chemical Industries).

in vitro γ -secretase assay

Human brain:

The DRM fraction (fraction 4 and 5 in figure 6B) of human brain was suspended in four-folds volume of buffer C (50 mM PIPES pH 7.0, 0.25 M sucrose, 1 mM EGTA) including 0.25% (w/v) CHAPSO and protease inhibitor cocktail (5 μ g/ml chymostatin (Peptide Institute, Osaka, Japan), 5 μ g/ml leupeptin (Peptide Institute) and 5 μ g/ml pepstatin A (Peptide Institute)), and subjected to centrifugation at 140,000 x g for 1h at 4°C. The resultant precipitates were further suspended in four-folds volume of buffer C including 0.25% [w/v] CHAPSO and mixed well by passing through into 30-gauge needle for 10 times. To the DRM suspension, purified C99/CTF β -FLAG was added to final 5 nM and incubated for 4 h at 37°C. The membrane suspension was centrifuged at 15,000 x g for 10 min. The supernatant including the γ -site-cleaved products was quantified for A β 40 and A β 42 with commercial sELISA kits (IBL, Co Ltd., Fujioka Japan). A β values are normalized against the amount of total protein.

Cells:

The DRM fraction (fraction 3 and 4 in figure 8A) of cells was suspended in three-folds volume of buffer H (20 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol) including 0.25% (w/v) CHAPSO and protease inhibitor cocktail, and subjected to centrifugation at 48,000 rpm for 1h at 4°C. The resultant precipitates were further suspended in buffer H including 0.25% (w/v) CHAPSO and mixed well by passing through into 30-gauge needle for 10 times. To the DRM suspension, Purified C99/CTF β -FLAG was added to final 0.45ng/ μ l and incubated for 2 h at 37°C with or without 10 μ M γ -secretase inhibitor DAPT. The DRM suspension was centrifuged at 48,000 rpm for 30 min,

and the supernatant including A β was quantified with commercial sELISA kits (IBL, Co Ltd., Fujioka Japan). A β values are normalized against the amount of total protein.

Mouse brain:

The DRM fraction of (fraction 5 in figure 11A, 14A, 16A) of mouse brain was suspended in three-folds volume of buffer H including 0.25% (w/v) CHAPSO and protease inhibitor cocktail, and subjected to centrifugation at 48,000 rpm for 1h at 4°C. The resultant precipitates were further suspended in 2 ml of buffer H including 0.25% (w/v) CHAPSO and mixed well by passing through into 30-gauge needle for 10 times. To the DRM suspension (protein concentration 100 μ g/ml), purified C99/CTF β -FLAG was added to final 0.9 ng/ μ l and incubated for 2 h at 37°C with or without 10 μ M γ -secretase inhibitor DAPT. The DRM suspension was centrifuged at 48,000 rpm for 30 min, and the supernatant including A β was quantified with commercial sELISA kits (IBL, Co Ltd., Fujioka Japan). A β values are normalized against the amount of total protein.

Western blotting

The samples were mixed with 5x Sample buffer and were separated by electrophoresis in a Tris-glycine SDS polyacrylamide discontinuous gel (8% (w/v) and 15% (w/v) polyacrylamide). After the electrophoresis, proteins were transferred to Nitrocellulose membrane in the transfer buffer by electrophoresis with 500mA for 3.5 h. The membrane was incubated in the 5% Skim milk in TBST (20 mM Tris, 137 mM NaCl, 0.05% Tween20) for blocking. After washing with TBST, the membrane was incubated with a primary antibody for overnight at 4°C. The membrane was incubated with a secondary antibody for 1 h at room temperature. After incubation, the membrane was washed with TBST and incubated with ECL Western Blotting Detection Reagents (GE healthcare). The protein detection was performed with LAS-4000 mini (Fuji film) and quantitated by ImageJ software.

Antibodies used in this study:

Mouse monoclonal antibody

- anti-flotillin-1 (BD Biosciences, San Jose, CA, USA)1/1,000

Rabbit polyclonal antibody

- anti-calnexin (Enzo Life Sciences, Farmingdale, NY, USA) 1/2,000
- anti-PS1 C-terminal (GIL3, gifted from Dr.Morishima)1/2,000
- anti-Pen-2 (Zymed Laboratories, South San Francisco, CA, USA).....1/1,0000
- anti-nicastrin (Sigma-Aldrich, St. Louis, MO, USA) 1/1,000

Secondary antibodies used in this study;

- anti-mouse IgG peroxidase-linked species-specific whole antibody (GE Health Care)
1/10000

- anti-rabbit IgG peroxidase-linked species-specific whole antibody (GE Health Care)
1/10000

Reagents

Buffer H: 20 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol

Buffer C: 50 mM PIPES pH 7.0, 0.25 M sucrose, 1 mM EGTA

PBS: 10 mM sodium phosphate buffer pH 7.4, 137 mM NaCl, 27mM KCl

Protease inhibitor (PI) mix: 5 µg/ml Leupeptin, 5 µg/ml Pepstatin A, 5 µg/ml Chymostatin

TBST: 20 mM Tris-HCl, 137 mM NaCl, 0.05% Tween20

TNE: 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA

5x SDS sample buffer: 43%(v/v) Glycerol, 16%(w/v) SDS, 64 ng/ml Bromo phenol blue, 5 mM EDTA, 0.22 M Tris-HCl pH 6.8

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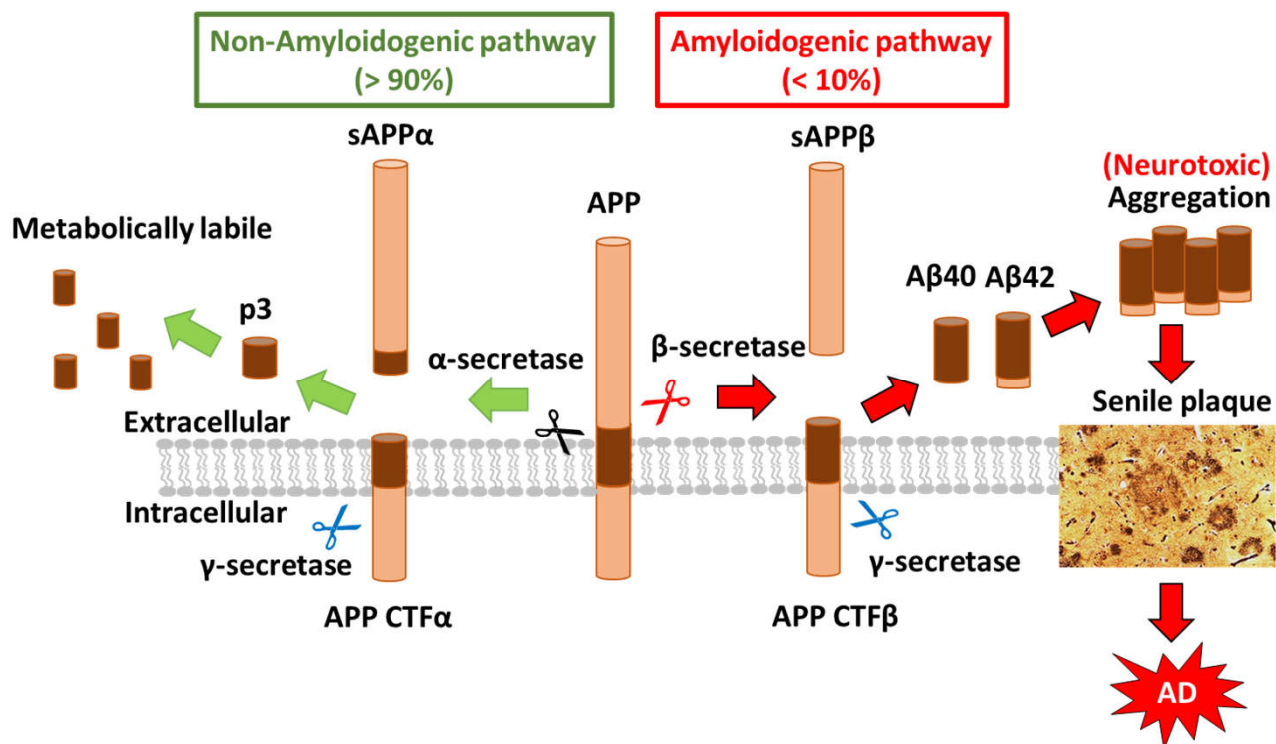


Figure 1. Pathogenesis of Alzheimer's disease (AD) and APP processing

Alzheimer's disease (AD) is a progressive neurodegenerative disorder with hallmarks of senile plaques and tangles in the brain. The production, aggregation, and accumulation of A β in the brain are initial steps in the pathogenesis of Alzheimer's disease. APP can undergo non-amyloidogenic pathway and amyloidogenic pathway. In the non-amyloidogenic pathway, APP is cleaved first by α -secretase to generate sAPP α . Then the CTF α is cleaved by γ -secretase to release p3 into extracellular, and AICD is retain inside the cell. In the amyloidogenic pathway, APP is cleaved first by β -secretase and generate sAPP β and CTF β . The CTF β is cleaved by γ -secretase to generate AICD inside the cell, and release A β into extracellular.

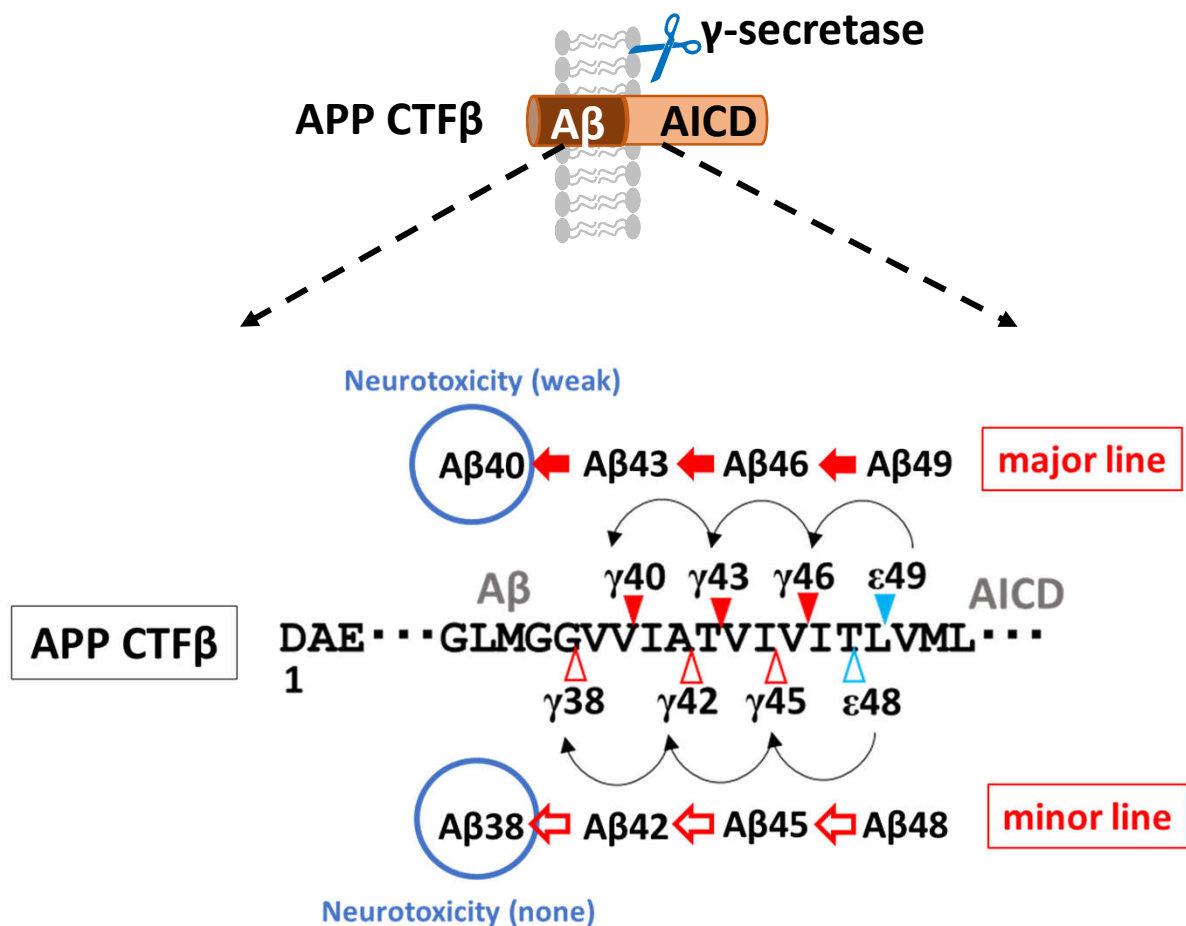


Figure 2. Aβ production lines

γ -secretase firstly cleaves APP CTFβ at ϵ -site to generate longer forms of Aβ, then cleaves at γ -site to release shorter forms of Aβ. The Aβ has two production line, the line convert Aβ49 to Aβ40 is the major line, and the line convert Aβ48 to Aβ38 is the minor line. The neurotoxicity of shorter forms of Aβ are weak or none neurotoxic.

γ -secretase complex

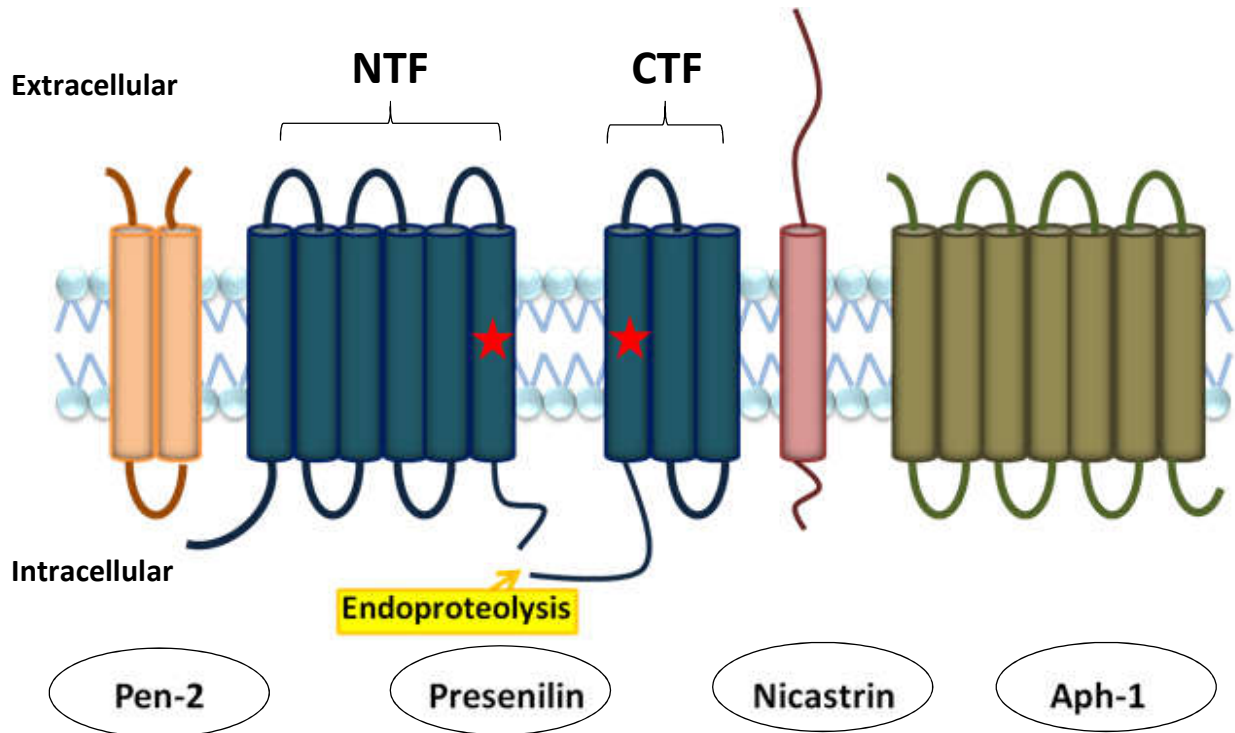


Figure 3. γ -Secretase complex

γ -Secretase complex is a key enzyme of $A\beta$ generation which is composed of PEN-2, presenilin, nicastrin and APh-1. Presenilin is the active component of the γ -secretase complex which is composed of two pieces, NTF and CTF.

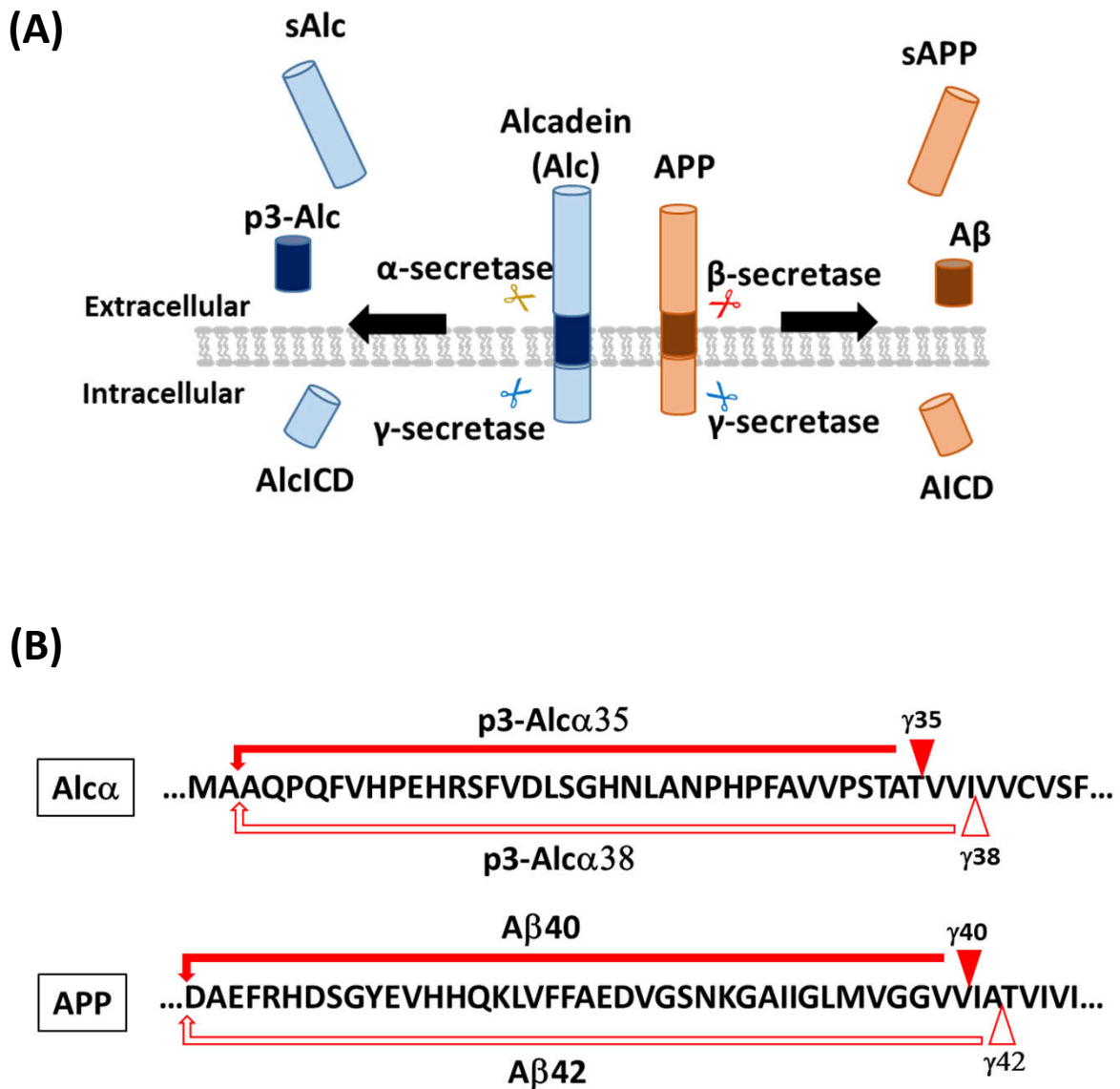


Figure 4. Metabolism of APP and Alc

In neurons, APP and Alc undergo parallel metabolic fates. Alcadin are cleaved by α -secretase. Subsequent cleavage of the remaining AlcCTF involves γ -secretase, and release extracellular peptide p3-Alc, and AlcICD is retain inside the cell (A). The same as APP, Alcadin can be cleaved by γ -secretase and produce major and minor products. In Alcadin α (Alc α), the p3-alc α 35 is the major γ -secretase cleavage product as is the A β 40 in APP, and p3-alc α 38 is the minor product as is the A β 42 in APP (B).

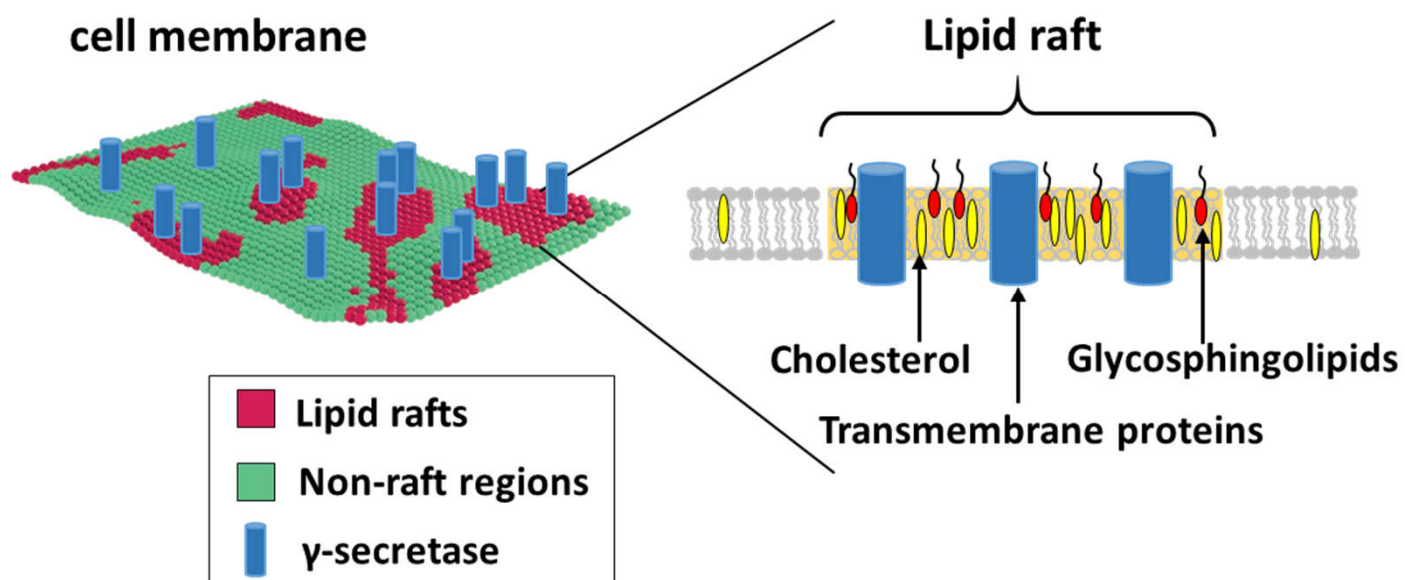


Figure 5. Structure of lipid raft

Lipid rafts, which γ -secretase are largely localized, are enriched of cholesterol and glycosphingolipids.

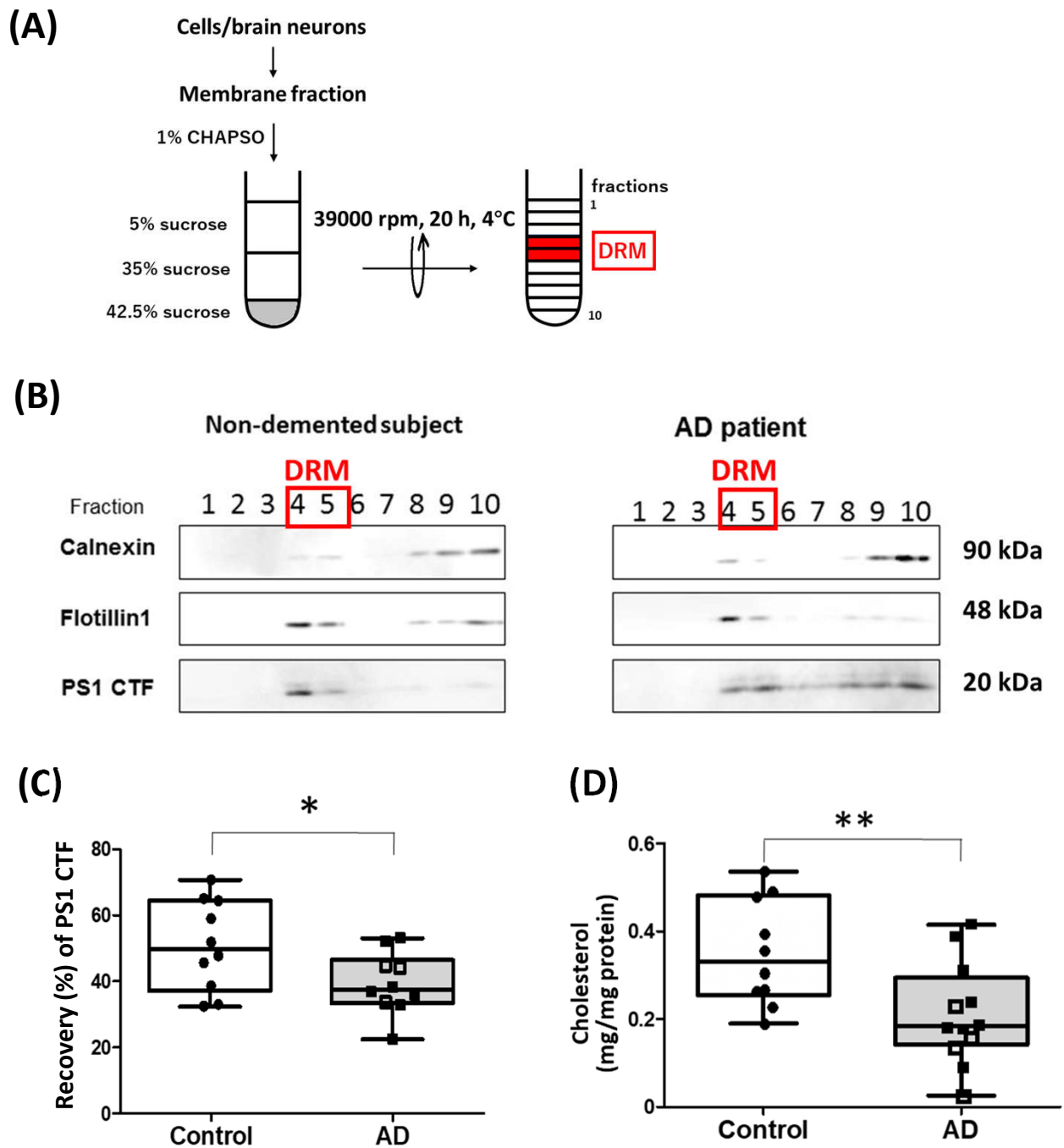
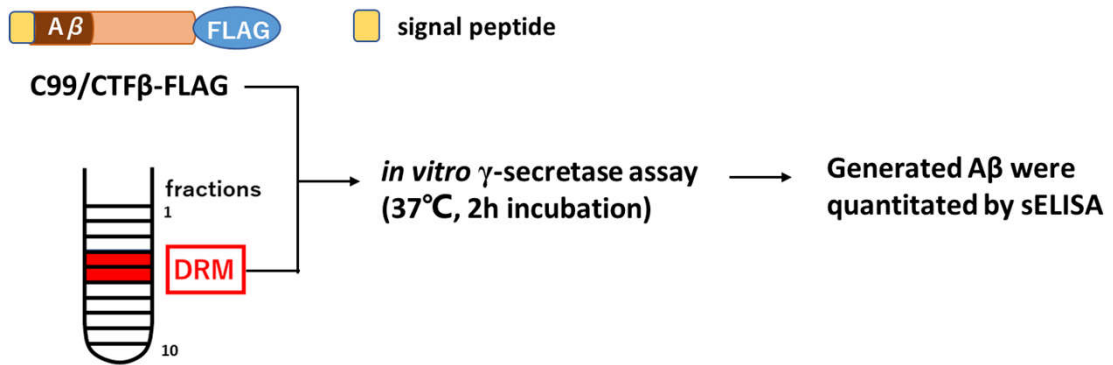


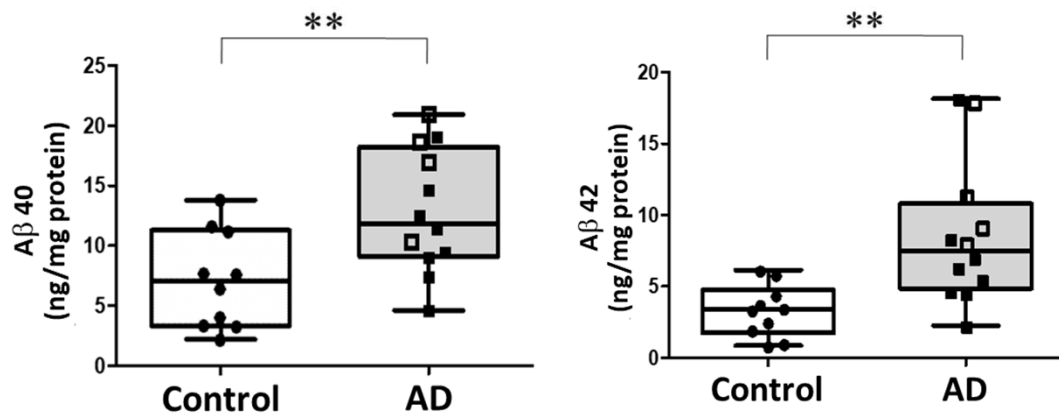
Figure 6. Cholesterol level and microlocalization of γ -secretase in DRM from human brain

- (A) DRM preparation.
- (B) Immunoblot analysis of microlocalization of γ -secretase components in different fractions along with Calnexin (non-DRM resident), Flotillin 1 (DRM resident). Fractions 4 and 5 were considered as DRM fraction.
- (C) Recovery of γ -secretase component, PS1 CTF, from DRM was quantitated.
- (D) Cholesterol level in DRM. (C-D) Open symbols indicate *APOE* $\epsilon 4$ carriers. Statistical analysis was performed using Student's t-test ($n = 10$ for non-AD controls and $n=12$ for AD subjects, mean $\pm$ S.E., * $P < 0.05$, ** $P < 0.01$).

(A)



(B)



(C)

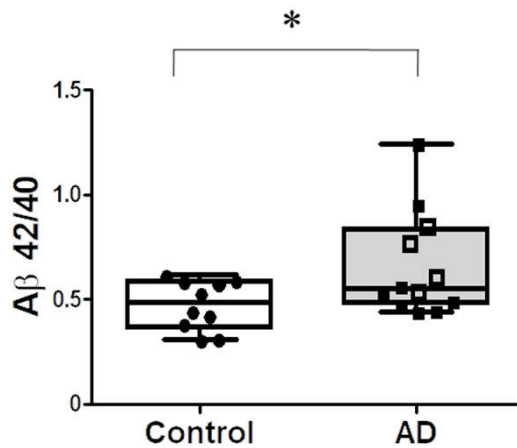


Figure 7. Aβ production by *in vitro* γ-secretase assay in DRM from human brain.

(A) *In vitro* γ-secretase assay.

(B) Production of Aβ40 and Aβ42 by *in vitro* γ-secretase assay.

(C) Ratio of Aβ42/40. (B-C) Open symbols indicate *APOE* ε4 carriers. Statistical analysis was performed using Student's t-test (n = 10 for non-AD controls and n=12 for AD subjects, mean ± S.E., *P < 0.05, **P < 0.01).

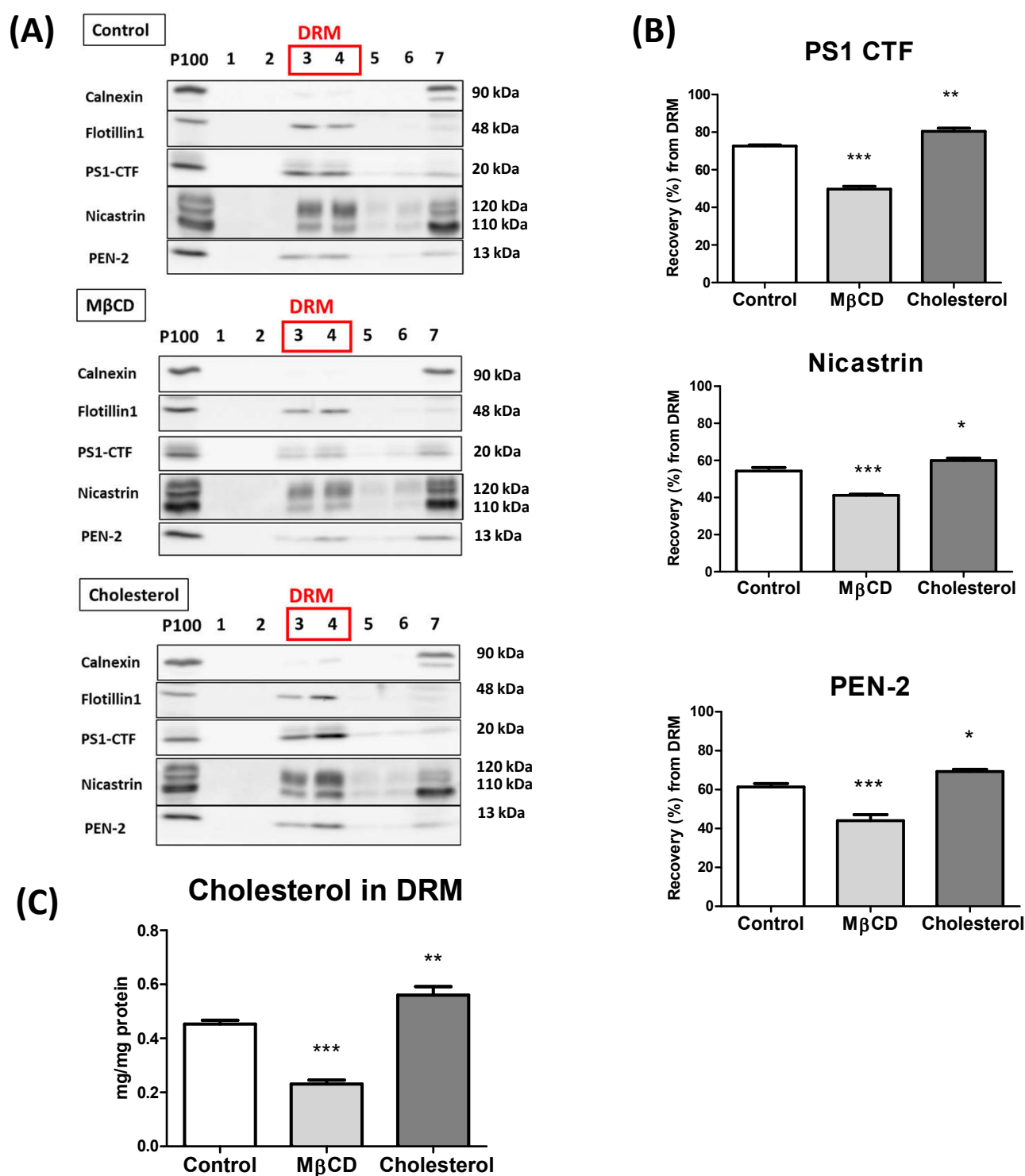


Figure 8. Cholesterol level and microlocalization of γ -secretase components in cells treated with MβCD or cholesterol-saturated MβCD.

- (A) Immunoblot analysis of microlocalization of γ -secretase components in different fractions along with Calnexin and Flotillin 1. Fractions 3 and 4 were considered as DRM fraction.
- (B) Recovery of γ -secretase components, PS1 CTF, Nicastrin and PEN-2, from DRM were quantitated.
- (C) Cholesterol level in DRM from cells. (B-C) Statistical analysis was performed using Dunnett's multiple comparison test (mean $\pm$ S.E., n = 5, *P < 0.05, **P < 0.01, ***P < 0.001).

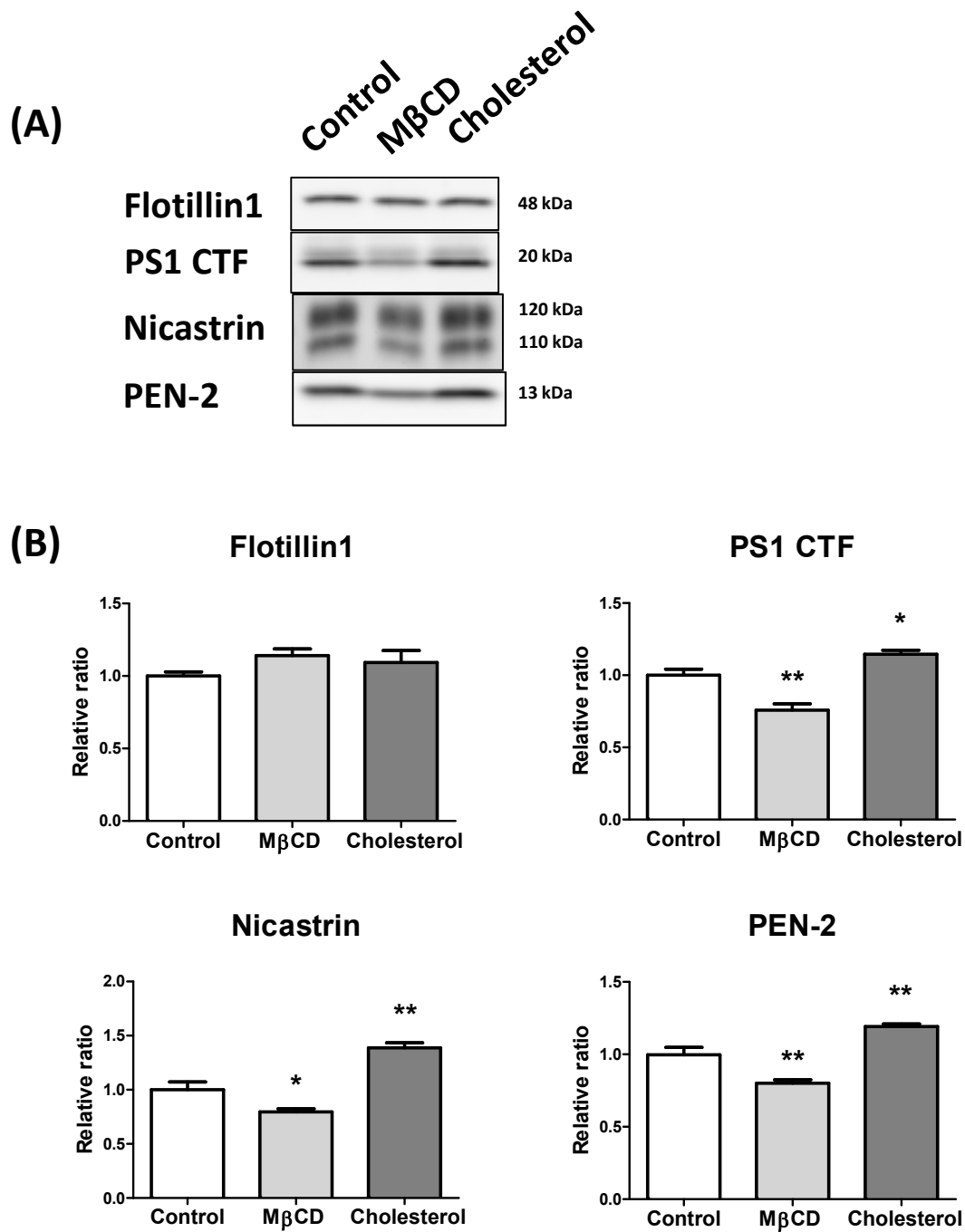


Figure 9. Concentration of γ -secretase components in DRM from cells treated with M β CD or cholesterol-saturated M β CD.

- (A) Immunoblot analysis of γ -secretase components in DRM along Flotillin 1.
- (B) Concentration of γ -secretase components, PS1 CTF, Nicastrin and PEN-2, along with Flotillin1 in DRM were quantitated. Statistical analysis was performed using Dunnett's multiple comparison test (mean $\pm$ S.E., n = 4, *P < 0.05, **P < 0.01, ***P < 0.001).

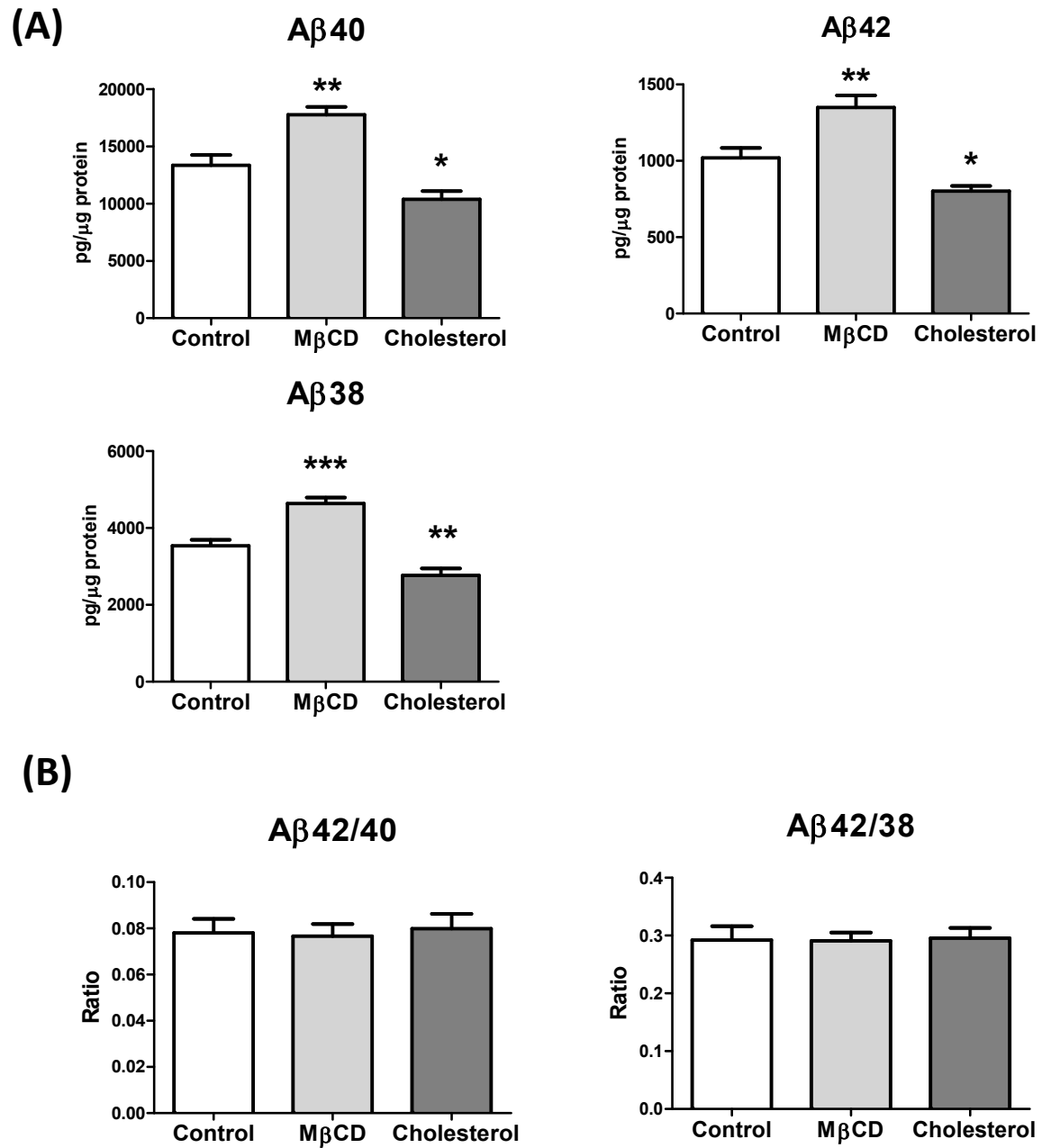
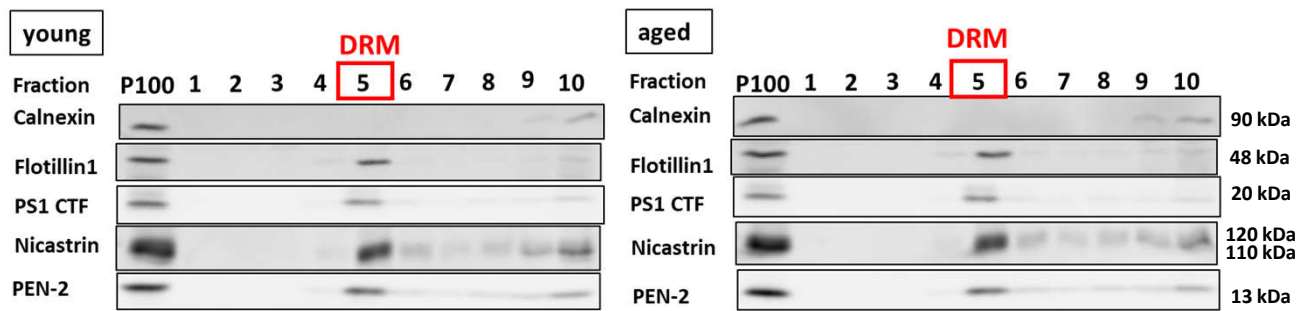


Figure 10. A β production by *in vitro* γ -secretase assay in DRM from cells treated with M β CD or cholesterol-saturated M β CD.

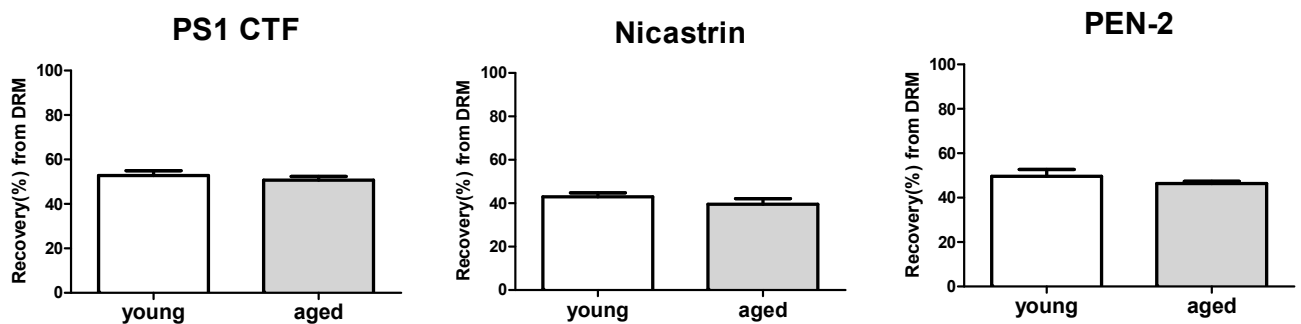
(A) Production of A β 40, A β 42 and A β 38 by *in vitro* γ -secretase assay.

(B) Ratio of A β 42/40 and A β 42/38. (A-B) Statistical analysis was performed using Dunnett's multiple comparison test (mean $\pm$ S.E., n = 8, *P < 0.05, **P < 0.01, ***P < 0.001).

(A)



(B)



(C)

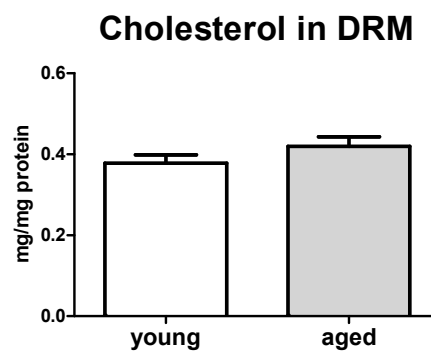


Figure 11. Cholesterol level and microlocalization of γ -secretase components in DRM from young and aged mouse neurons.

- (A) Immunoblot analysis of microlocalization of γ -secretase components in different fractions along with Calnexin and Flotillin 1. Fraction 5 was considered as DRM fraction.
- (B) Recovery of γ -secretase components, PS1 CTF, Nicastrin and PEN-2, from DRM were quantitated.
- (C) Cholesterol level in DRM. (B-C) Statistical analysis was performed using Dunnett's multiple comparison test (mean $\pm$ S.E., n = 3).

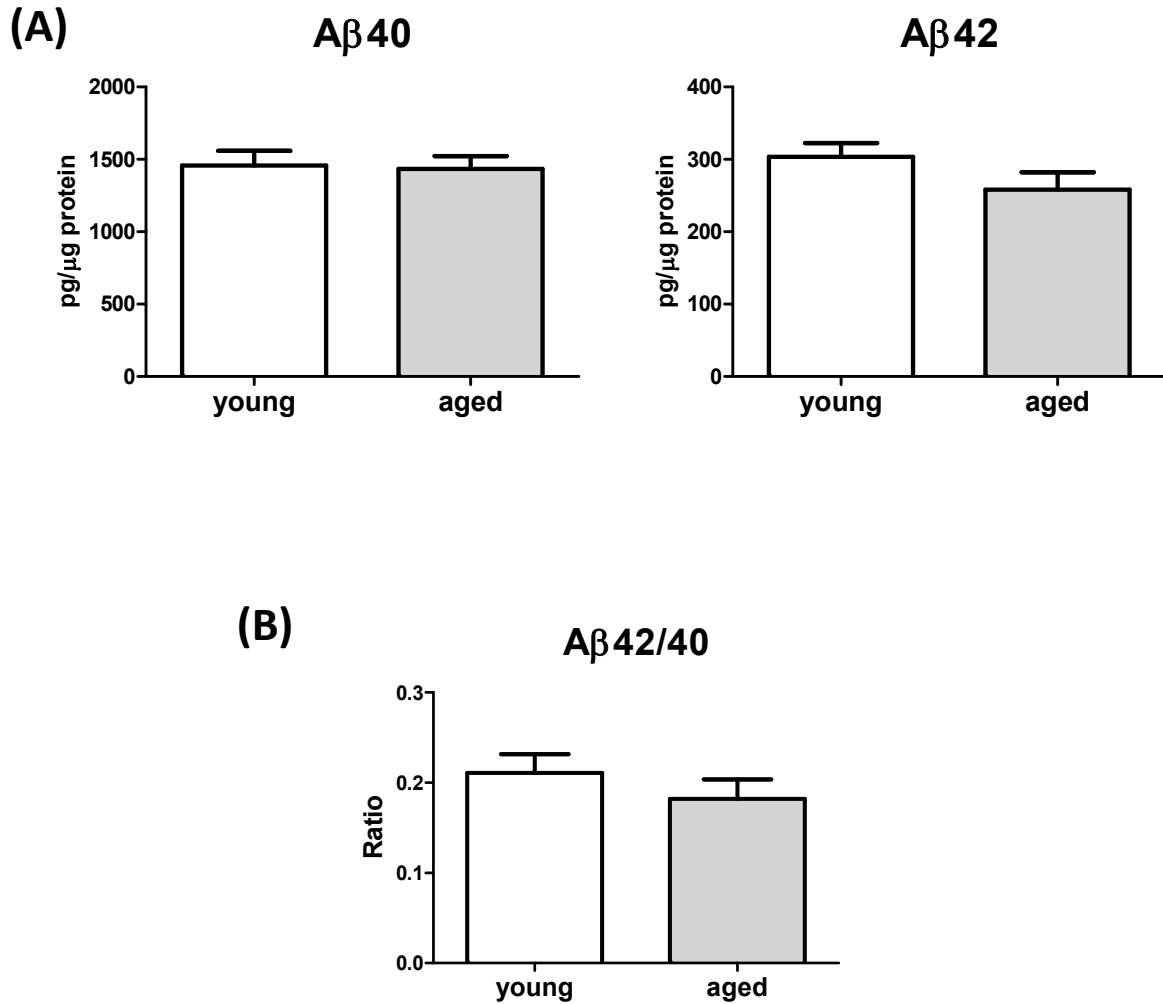


Figure 12. A β production by *in vitro* γ -secretase assay in DRM from young and aged mouse neurons.

(A) Production of A β 40 and A β 42 by *in vitro* γ -secretase assay.

(B) Ratio of A β 42/40. (A-B) Statistical analysis was performed using Dunnett's multiple comparison test (mean $\pm$ S.E., n =3).

(A)

	Control diet	Low-fat diet	High-fat diet
Fat (gm%)	10	5	35
Cholesterol (mg/kg)	48	0	301

(B)

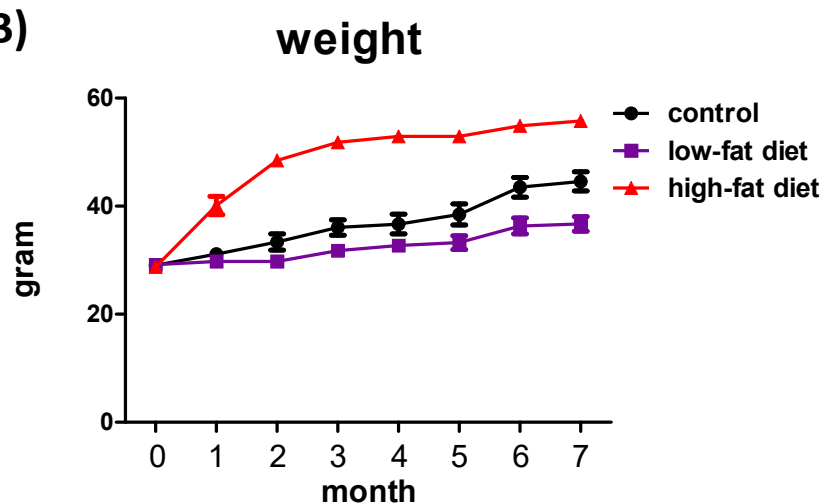


Figure 13. Influence of diet to mice

(A) The content of fat and cholesterol in different diets.

(B) Weight of mice fed with different diets.(mean $\pm$ S.E., control and low-fat diet n=4, high-fat diet n=3).

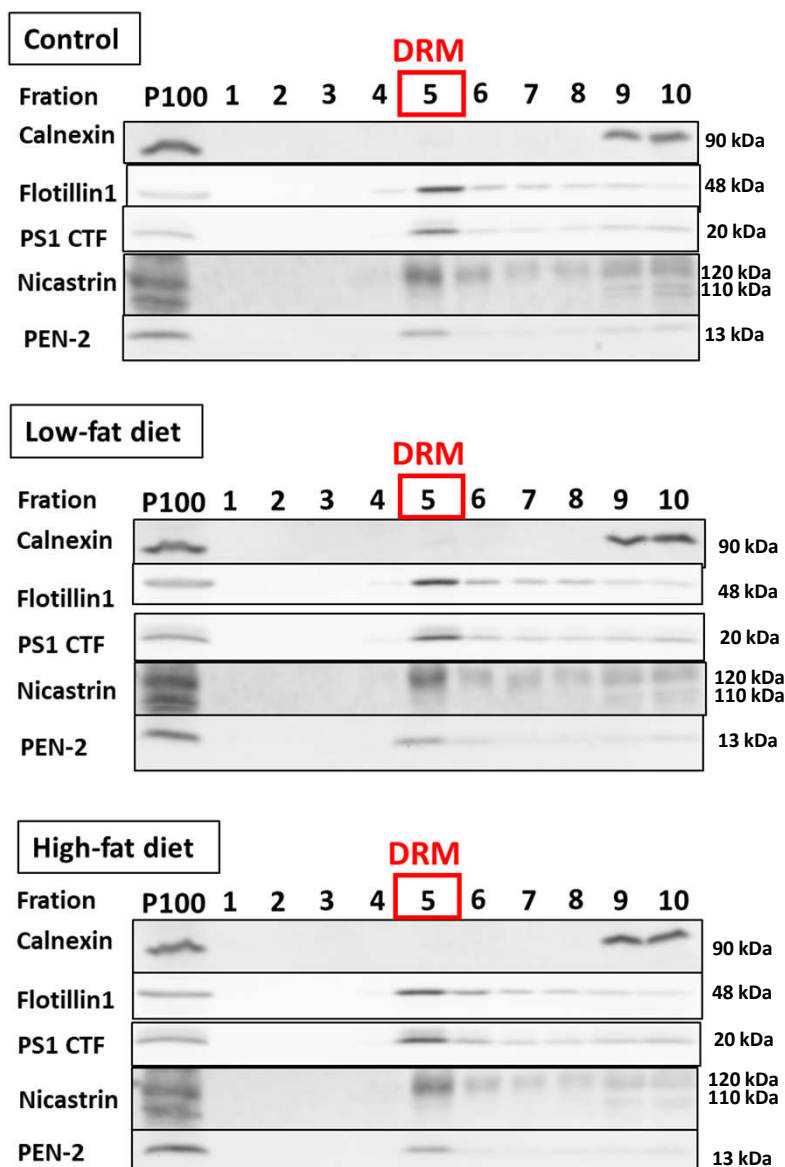
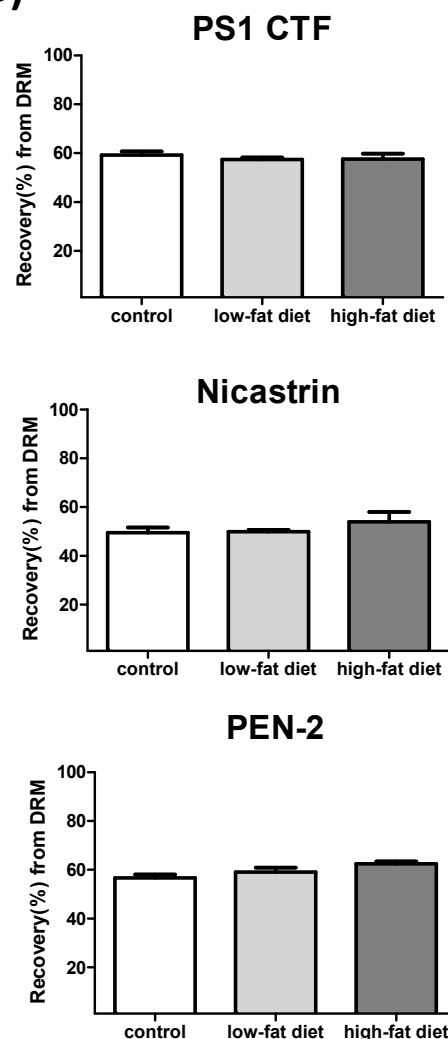
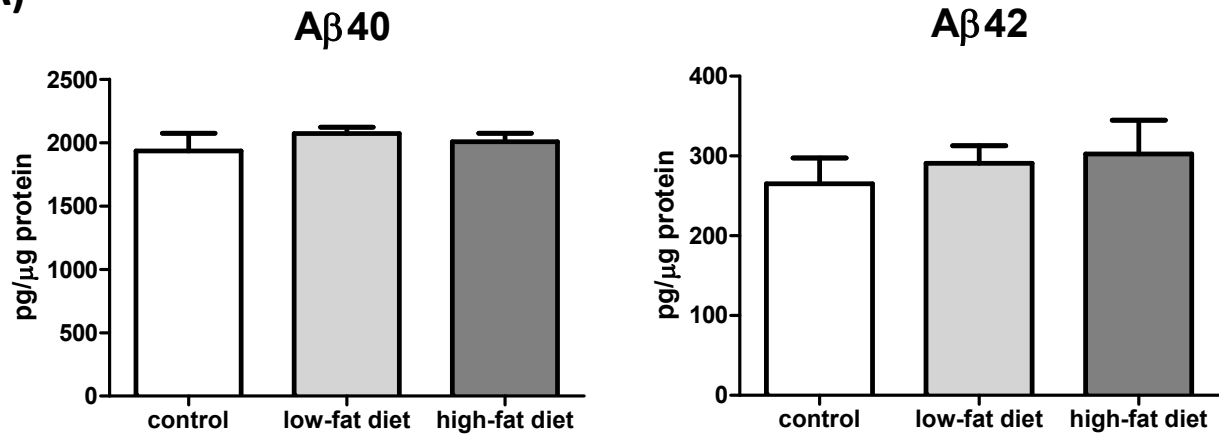
(A)**(B)****(C)**

Figure 14. Cholesterol level and microlocalization of γ -secretase components in DRM from neurons of mice fed with different diets.

- (A) Immunoblot analysis of microlocalization of γ -secretase components in different fractions along with Calnexin and Flotillin 1. Fraction 5 was considered as DRM fraction.
- (B) Recovery of γ -secretase components, PS1 CTF, Nicastrin and PEN-2, from DRM were quantitated.
- (C) Cholesterol level in DRM. (B-C) Statistical analysis was performed using Dunnett's multiple comparison test (mean $\pm$ S.E., control and low-fat diet n=4, high-fat diet n=3).

(A)



(B)

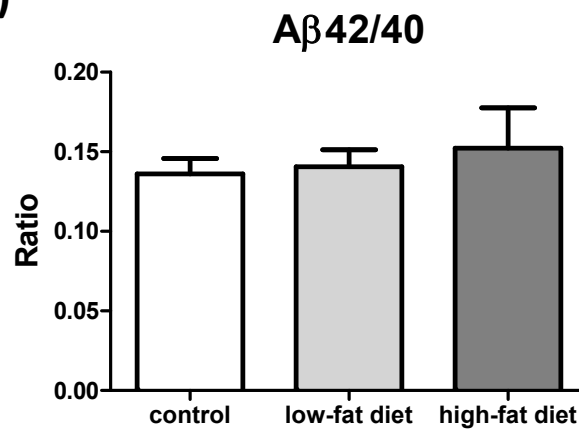


Figure 15. Aβ production by *in vitro* γ-secretase assay in DRM from neurons of mice fed with different diets.

(A) Production of Aβ40 and Aβ42 by *in vitro* γ-secretase assay.

(B) Ratio of Aβ42/40. (A-B) Statistical analysis was performed using Dunnett's multiple comparison test (mean ± S.E., control and low-fat diet n=4, high-fat diet n=3).

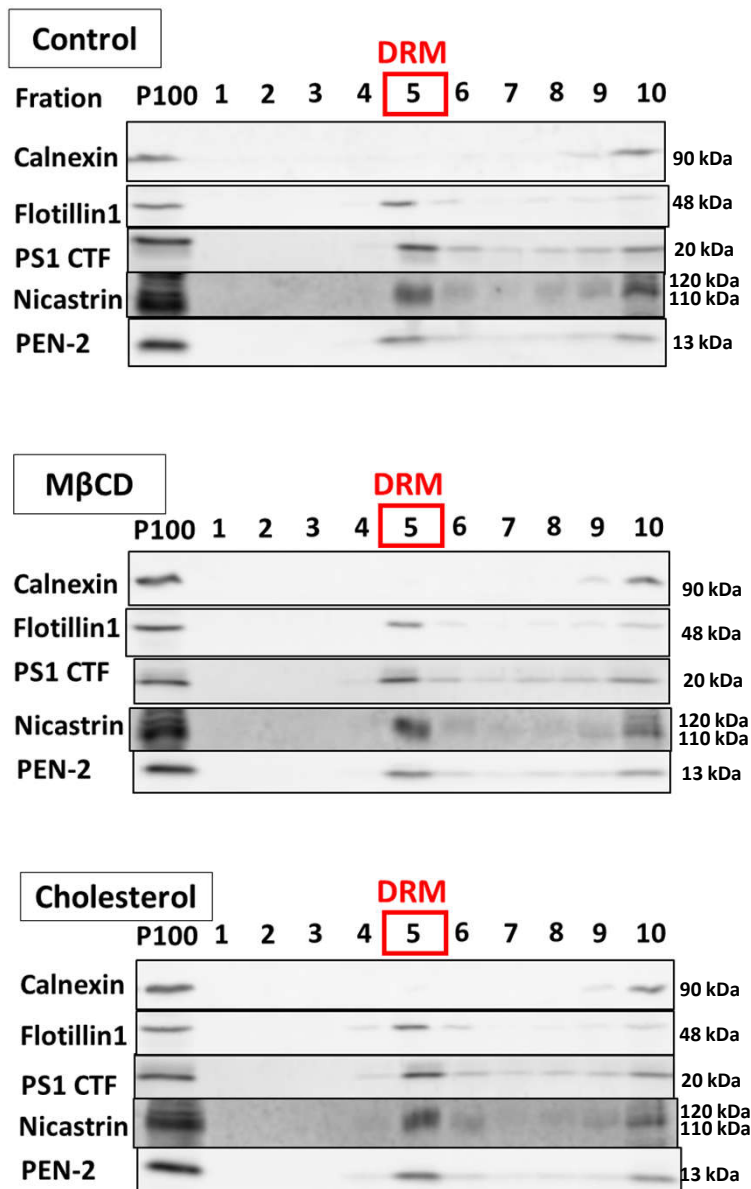
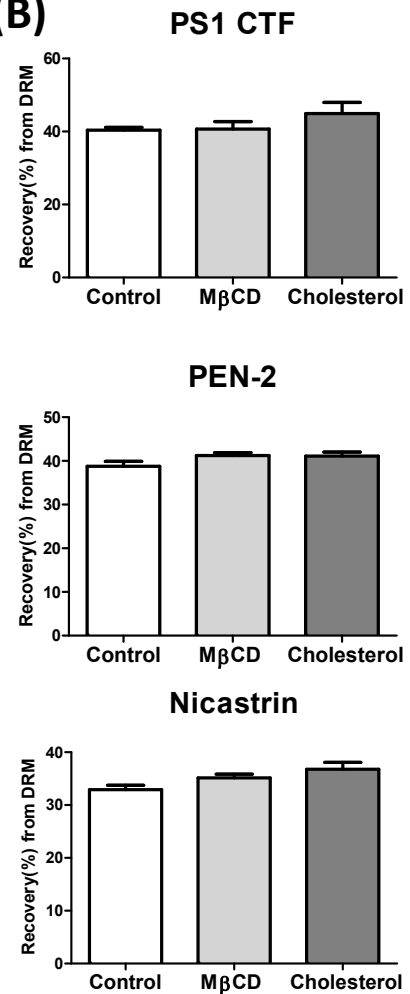
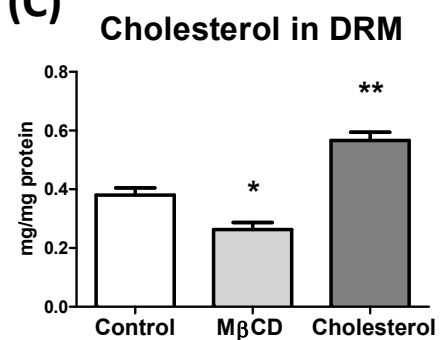
(A)**(B)****(C)**

Figure 16. Cholesterol level and microlocalization of γ -secretase components in DRM from mouse neurons with altered cholesterol levels.

- (A) Immunoblot analysis of microlocalization of γ -secretase components in different fractions along with Calnexin and Flotillin 1. Fraction 5 was considered as DRM fraction.
- (B) Recovery of γ -secretase components, PS1 CTF, Nicastrin and PEN-2, from DRM were quantitated.
- (C) Cholesterol level in DRM. (B-C) Statistical analysis was performed using Dunnett's multiple comparison test (mean $\pm$ S.E., n = 3, *P < 0.05, **P < 0.01).

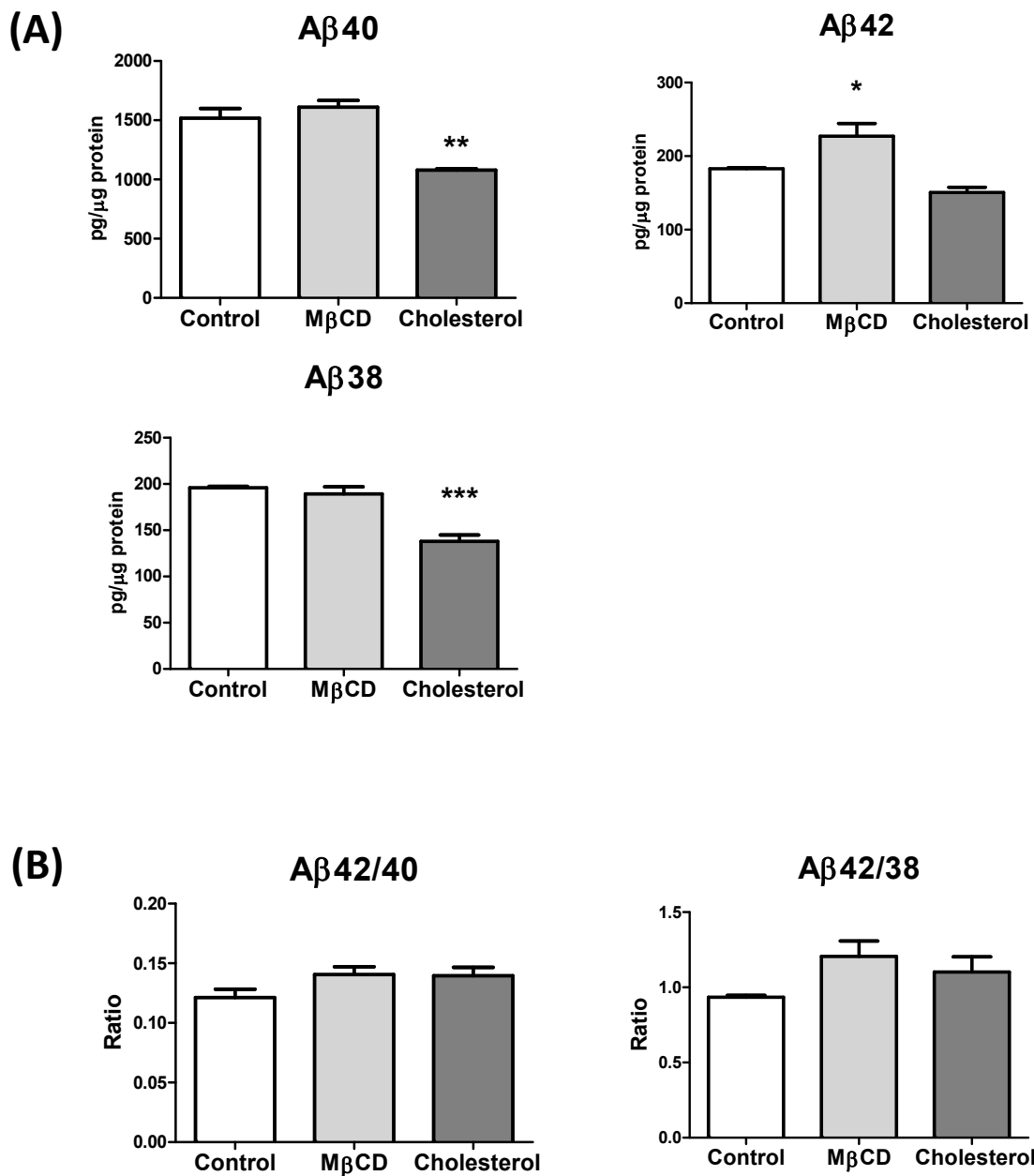
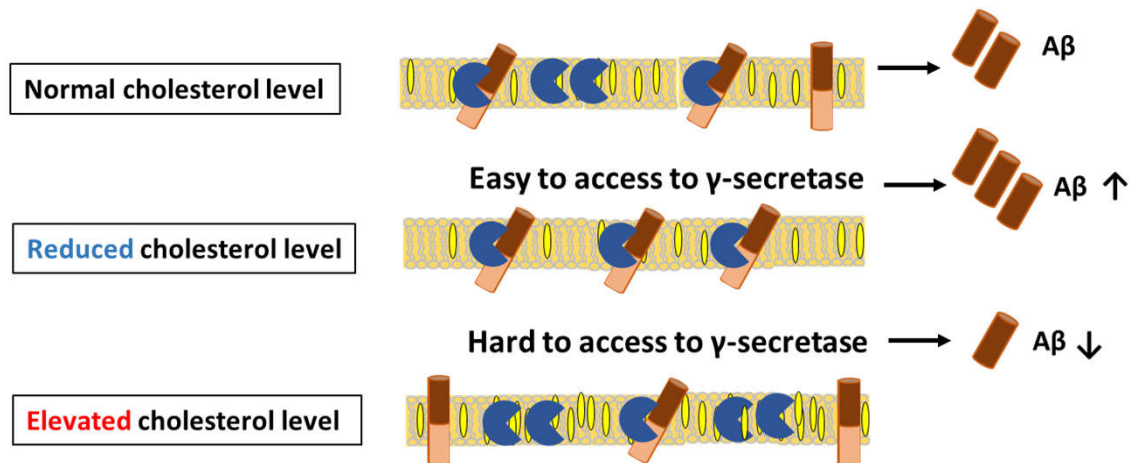


Figure 17. A β production by *in vitro* γ -secretase assay in DRM from mouse neurons with altered cholesterol levels.

(A) Production of A β 40, A β 42 and A β 38 by *in vitro* γ -secretase assay.

(B) Ratio of A β 42/40 and A β 42/38. (A-B) Statistical analysis was performed using Dunnett's multiple comparison test (mean $\pm$ S.E., n = 3, *P < 0.05, **P < 0.01, ***P < 0.001).

(A)



(B)

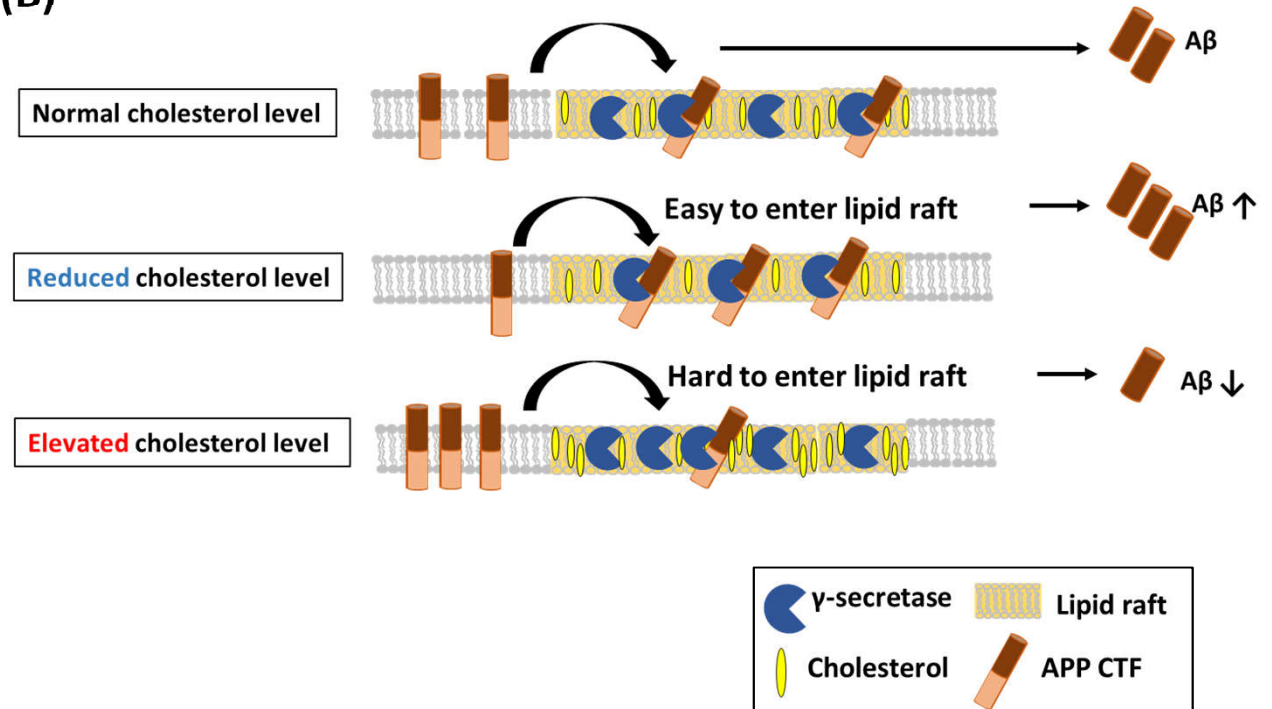


Figure 18. Possible mechanisms

Altered cholesterol levels possibly change the structure of lipid rafts, which results in altered ability of APP CTF accessing to γ -secretase complex (A) or entering the lipid rafts (B).

Table 1. Summary of subject information

	Age	Gender	ApoE	CDR	Senile plaque	NFT	Brain weight (g)	Neuropathological Diagnosis
Normal	81	M	2/3	0	0	1	1170	Unremarkable
Normal	84	F	3/3	0	1	1	1030	Unremarkable
Normal	78	F	3/3	0	1	2	1133	CVD
Normal	82	F	3/3	0	0.5	1	1125	CVD
Normal	82	F	3/3	0	0	2	1015	Myeloradiculopathy, multiple compression
Normal	80	M	3/3	0	0.5	2	1220	Unremarkable
Normal	82	M	3/3	0	1	1	1250	CVD
Normal	78	M	2/3	0	0	1	1325	Unremarkable
Normal	78	M	3/3	0	0	1	1272	Unremarkable
Normal	82	M	3/3	0	1	1	1212	Unremarkable
AD	81	F	3/3	≥1	3	4 or 5	1127	AD
AD	79	F	3/3	1	3	5	1100	AD/ CVD
AD	74	M	3/4	1	3	5	1149	AD/ CVD
AD	84	M	3/3	2	3	4	1431	AA/ AD
AD	76	M	2/3	3	3	6	1254	AD/ AA
AD	82	F	3/3	3	3	6	1131	AD/ CVD
AD	82	F	3/3	3	3	5	1123	AD
AD	82	F	3/3	3	3	5	1243	AD/ AA/ PSP
AD	84	M	3/4	3	3	5	1320	AD
AD	75	M	4/4	3	3	6	1046	AD
AD	81	M	3/3	1 or 2	3	6	1450	AD
AD	70	M	4/4	≥1	3	4	1082	AD

Details from twelve AD patients and ten non-demented subjects are shown. Staging of senile plaque and NFT was according to Braak's criteria (Braak & Braak 1991; Braak 2006). CDR, clinical dementia rating. CVD, cerebral vascular disease; AA, amyloid angiopathy; PSP, progressive supranuclear palsy.

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