Age-related modulation of γ-secretase activity in non-human primate brains

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Abbreviations used: AD: Alzheimer's disease; Aβ: amyloid-β peptide; APP: amyloid-β precursor protein; PS1: presenilin-1; PS2: presenilin-2; APH-1a: anterior pharynx-defective-1a; PEN-2: presenilin enhancer-2; ApoE: apolipoprotein E; TBS: Tris-buffered saline; CHAPSO: 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; DAPT: *N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine t-butyl ester

Abstract

Age-dependent accumulation of the amyloid-β peptide (Aβ) in the brain is a precondition for development of Alzheimer's disease. A relative increase in the generation of longer Aβ species such as Aβ42 and Aβ43 is critical for Aβ deposition, but the underlying mechanism remains unresolved. Here, we performed a cell-free assay using microsome fractions of temporal cortex tissues from 42 cynomolgus monkeys and found that Aβ40-generating γ-secretase activity (γ40) decreased with age, whereas Aβ42-generating γ-secretase activity (γ42) was unaltered. In ELISAs, more than 80% of monkeys over 20 years old showed evidence of Aβ accumulation in the temporal cortex. The ratio of γ42 to γ40 increased with age and correlated with the level of accumulated Aβ. These results suggest that γ-secretase activity undergoes age-related, non-genetic modulation and that this modulation may cause Aβ accumulation in aging brains. Similar modulation may predispose aged human brains to Alzheimer's disease.

Keywords: Alzheimer's disease; amyloid-β peptide; γ-secretase; aging; cynomolgus monkey

Running title: Age-related modulation of γ-secretase

Introduction

The prevalence of Alzheimer's disease (AD) increases exponentially from the age of 65 (Jorm *et al.* 1987). Accordingly, aging is recognized as a non-genetic risk factor for AD. AD is neuropathologically characterized by widespread appearance of extracellular amyloid plaques and intracellular neurofibrillary tangles that are composed of amyloid β-peptide (Aβ) and hyperphosphorylated tau protein respectively. Although both proteins are implicated in the pathogenic mechanism, Aβ is thought to act upstream of tau (Hardy & Selkoe 2002). Deposition of Aβ in the brain begins decades prior to the manifestation of the clinical symptoms of AD (Price *et al.* 2009). Biochemical studies using consecutive autopsy brains indicate that Aβ accumulation is present in more than 50% of elderly individuals (Funato *et al.* 1998). Although the amyloid burden in the aged brain does not always represent a preclinical or early stage of AD, recent neuroimaging studies reveal that high retention of amyloid-binding compounds in the brain is associated with longitudinal cognitive decline (Storandt *et al.* 2009, Villemagne *et al.* 2011).

Aβ is produced in neurons by sequential proteolysis of the amyloid-β precursor protein (APP) by β- and γ-secretases. The γ-secretase cleavage at multiple sites generates several Aβ species with different C-terminal lengths. Although the molecular mechanisms underlying Aβ deposition in the brain remain unresolved, several lines of evidence underscore the significance of longer species Aβ42 and Aβ43. Indeed,

Aβ42 and Aβ43 are the initially deposited, predominant Aβ species in the brains of AD patients, whereas Aβ40 is the major product under physiological conditions (Iwatsubo *et al.* 1994, Saito *et al.* 2011). AD-causing mutations in presenilin-1 (PS1) and presenilin-2 (PS2) genes, which encode the catalytic components of the γ-secretase complex, increase the relative level of Aβ42 generation, but do not always increase the total activity of γ-secretase (Bentahir *et al.* 2006). Transgenic mice overexpressing an artificial fusion transgene selectively yielding Aβ42 developed age-dependent Aβ deposition in the brain, whereas mice similarly overexpressing Aβ40 did not (McGowan *et al.* 2005).

Aggregation of Aβ in the brain and brain vulnerability to Aβ toxicity is age- and species-dependent (Geula *et al.* 1998). Age-related amyloid burden in the brain and cognitive decline was observed in non-human primates, and the morphology, distribution and chemical composition of amyloid plaques in aged monkeys display close similarities to those observed in aged humans (Wisniewski *et al.* 1973, Podlisny *et al.* 1991, Nakamura *et al.* 1995, Sani *et al.* 2003, Nagahara *et al.* 2010). To study the temporal profile of Aβ accumulation in the monkey brain and to test the hypothesis that modulation of γ-secretase activity causes Aβ deposition in aged brains, we investigated Aβ accumulation and γ-secretase activity in the brains of cynomolgus monkeys of various ages. The use of monkey brains allowed us to overcome the limitations involved in using human autopsy brains. This includes the fact that several

medicines, including non-steroidal anti-inflammatory drugs and fenofibrate, and agonal states such as prolonged hypoxia, acidosis and fever, can potentially modulate γ-secretase activity to alter the Aβ42-generating ratio (Kukar *et al.* 2005, Quintero-Monzon *et al.* 2011). Here, using cynomolgus brains, we found that the ratio of Aβ42 generation increased in an age-dependent manner and correlated with Aβ deposition.

Materials and methods

Brain samples

Temporal cortex tissues from 42 cynomolgus monkeys (*Macaca fascicularis*, 4–36 years of age) were used. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Shiga University of Medical Science and the National Institute of Biomedical Innovation, and were performed according to the Guide for the Care and Use of Laboratory Animals. All monkeys were housed in individual cages and maintained according to guidelines for experimental animal welfare. Six monkeys died naturally. The remaining animals were killed under deep pentobarbital anesthesia as previously described (Kimura *et al.* 2003). No monkeys were subjected to any specific pharmacological treatment for at least 6 months prior to death. Tissues were snap-frozen and stored until use.

Immunohistochemistry

Sections of formalin-fixed, paraffin-embedded brain tissue (6 μm thick) were used for immunostaining as previously described (Nakamura et al. 1995). The primary antibodies used were mouse monoclonal antibodies against the C-terminus of Aβ42 (BC05; WAKO Pure Chemicals, Osaka, Japan), the C-terminus of Aβ40 (BA27; WAKO), residues 25–35 of human Aβ (BS85; WAKO) and the N-terminus of human Aβs (82E1; Immuno-Biological Laboratories, Gunma, Japan) and rabbit polyclonal antibodies against the C-terminus of human Aβ40 or Aβ42 (Immuno-Biological Laboratories). The sections were counterstained with hematoxylin.

Measurement of brain Aβ

Frozen tissues from monkey temporal cortices were homogenized using a motor-driven Teflon/glass homogenizer (10 strokes) in four volumes of Tris-buffered saline (TBS: 20 mM Tris, pH 7.5, 150 mM NaCl, 0.5 mM EDTA) that contained a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The homogenates were centrifuged at 100,000 × *g* for 20 min on a TLA 100.4 rotor in a TLX ultracentrifuge (Beckman, Palo Alto, CA). The supernatant was used as the soluble fraction. The pellet was lysed by brief sonication in an initial volume of 6 M guanidine hydrochloride in 50 mM Tris, pH 7.5, and then centrifuged at 100,000 × *g* for 10 min. The supernatant was diluted at 1:12 and used as the insoluble

fraction. The soluble and insoluble fractions were subjected to a DC protein assay (BioRad, Hercules, CA) and ELISAs specific for human Aβ40 and Aβ42 (WAKO Pure Chemicals), as the predicted amino acid sequence of the neuronal isoform of cynomolgus APP is completely homologous to that of humans (Podlisny et al. 1991).

Cell-free assay for γ-secretase activity

The post-nuclear supernatants from the brain homogenates were centrifuged at 100,000 × *g* for 1 h. The membrane pellets were washed with HEPES buffer (50 mM HEPES, pH 7.0, 150 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$) and subsequently lysed in a lysis buffer containing 1% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid (CHAPSO). Solubilized γ-secretase was recovered by centrifugation at 100,000 × *g* for 30 min, and the concentrations of protein and CHAPSO were adjusted to 0.25 mg/mL and 0.25% w/v, respectively. The generation of Aβs in a mixture of solubilized γ-secretase and a recombinant human APP C-terminal fragment of 99 amino acids (C99) has been described previously (Mitsuishi *et al.* 2010). Briefly, CHAPSO-solubilized γ-secretase was incubated for 6 h at 37°C with the recombinant APP-C99-Flag substrate in the presence of 0.1% phosphatidyl choline. The concentrations of Aβ40 and Aβ42 were measured by ELISAs. Background was defined as the Aβ40 and Aβ42 levels in reaction mixtures in the presence of 1 μM

N-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine t-butyl ester (DAPT; Calbiochem, San Diego, CA). Values presented represent the mean ± SD of three independent reactions. Values for Aβ40- and Aβ42-generating γ-secretase activity (γ40 and γ42) represent background-subtracted Aβ40 and Aβ42 levels, respectively.

Immunoblotting

Membrane fractions of brain homogenates were lysed in a lysis buffer containing 1% NP40 and were subjected to immunoblotting as previously described (Mitsuishi et al. 2010). The following antibodies were used: anti-PS1 (Chemicon, Temecula, CA; MAB5232), anti-PS2 (Cell Signaling Technology, Danvers, MA), anti-nicastrin (Sigma-Aldrich, St. Louis, MO; N1660), anti-anterior pharynx-defective-1a (APH-1a) L/S (Covance, Berkley, CA), anti-presenilin enhancer-2 (PEN-2) (Calbiochem) and anti-β-actin (Sigma-Aldrich). The intensity of protein bands was quantified using the Image J software (NIH, Bethesda, MD) and normalized by the density of the β-actin band.

Statistical analysis

Correlation analyses were performed using the Spearman's rank correlation test. StatPlus:mac LE software (AnalystSoft, Vancouver, Canada) was used for statistical analyses. Values are reported as the mean ± SD. Probability (p) values < 0.05 were considered statistically significant.

Results

Age-related increases in Aβ accumulation

Histological examination of the temporal cortex, which is vulnerable to Aβ burden in both monkeys and humans (Sani et al. 2003), confirmed that the number of amyloid plaques increased with aging in cynomolgus monkeys. Immunohistochemical analysis revealed the occurrence of Aβ40-positive and Aβ42-positive amyloid plaques in 76% (16/21 cases) of monkeys over 21 years old. In all 16 cases, Aβ42-positive plaques were predominant over Aβ40-positive plaques (Fig. 1a).

We measured Aβ40 and Aβ42 levels in TBS-soluble and insoluble (guanidine hydrochloride-soluble) fractions from temporal cortex homogenates. There was an age-dependent increase in the combined levels of Aβ40 or Aβ42 in both fractions from monkeys over 21 years old (Fig. 1b and c). In accordance with the immunohistochemical observations, the level of Aβ42 was higher than that of Aβ40 in every Aβ-accumulated brain. High levels of Aβ42 (> 100 pmole/g of total protein) were detected in monkeys as young as 21 years of age and in 86% (18/21 cases) of monkeys over 21 years old. Accumulation of Aβ40 was observed only in brains with a considerable level of Aβ42 accumulation (>1,000 pmole/g of total protein), and the level of accumulated Aβ40 exhibited a linear correlation with that of Aβ42 (Fig. 1d).

These results suggest that Aβ42 precedes Aβ40 in accumulation.

Aβ concentration in the soluble fraction was less than 5% of that in the insoluble fraction. Levels of Aβ42 and Aβ40 in both fractions started to increase between 21 and 25 years of age (Fig. 2a–d). Increase in soluble Aβ40 or Aβ42 was exclusively observed in brains that exhibited considerable accumulation of the insoluble Aβ42 (>1,000 pmole/g protein) (Fig. 2e and f), whereas increase in soluble Aβs was coincident with increase in insoluble Aβ40 (Fig. 2g and h). Our cross-sectional study suggests that the increase in soluble Aβs follows the accumulation of insoluble Aβ42. There was no difference in the degree of Aβ accumulation between sexes (data not shown).

Cell-free assay for γ-secretase activity using brain microsome fractions

We examined whether frozen tissues of monkey brain were applicable for the cell-free γ-secretase activity assay. The amount of Aβ40 and Aβ42 generated by CHAPSO-solubilized γ-secretase from microsome fractions of cerebrocortical tissue from two young monkeys (7 years old; Aβ40: 434.62 ± 27.08 and 375.99 ± 13.32 pmole/g protein; Aβ42: 157.02 ± 9.21 and 114.39 ± 5.01 pmole/g protein) was equivalent to that generated by the γ-secretase from microsome fractions of cultured HEK293 cells (Aβ40: 373.39 ± 7.29 pmole/g protein; Aβ42: 123.09 ± 7.17 pmole/g protein). The generation of Aβ was sensitive to the γ-secretase inhibitor, DAPT. However, the reaction mixtures after incubation at 4°C or in the presence

of DAPT contained levels of Aβ species (at 4°C; Aβ40: 3.23 ± 1.23 and 50.10 ± 1.23 pmole/g protein; Aβ42: 21.16 ± 0.39 and 24.12 ± 0.71 pmole/g protein), which varied from brain to brain and paralleled Aβ levels in the solubilized γ-secretase preparations. Hence, we assumed that these background levels of Aβ were extracted from microsome membrane and/or Aβ aggregates in microsome fractions of cortical tissues. The γ42/γ40 ratios from the cynomolgus monkey brains (0.303 ± 0.025 and 0.293 ± 0.016) were equivalent to that of HEK293 cells (0.303 ± 0.004).

Age-related modulation of γ-secretase activity

Cortical tissues from the same frozen blocks used for Aβ quantification were used in a cell-free assay for Aβ generation. This assay revealed a negative correlation between γ40 and age (r^2=0.1600, p=0.009), but not between γ42 and age (Fig. 3a and b). The relationship between γ40 and age was qualitatively similar in female (n=26, r^2=0.0989, p=0.065; Fig. 3c) and male (n=16, r^2=0.2038, p=0.045; Fig. 3d) monkeys. The γ42/γ40 ratio was distributed within a range of 0.18–0.33 in monkeys between 4 and 20 years old and became higher as age increased to 20 years (Fig. 3e). The γ42/γ40 ratio correlated with age (r^2=0.3946, p=0.00001; Fig. 3e) and the logarithm of Aβ42 content in the brain lysate (r^2=0.48762, p=0.00000; Fig. 3f).

Expression levels of γ-secretase components in brains

We compared the expression levels of γ-secretase complex components in aged monkeys with a high γ42/γ40 ratio (n=6, mean age=33.7 ± 2.4 years, mean γ42/γ40 ratio=0.437 ± 0.042) to those in young monkeys with a low γ42/γ40 ratio (n=6, mean age=5.5 ± 1.5 years, mean γ42/γ40 ratio=0.258 ± 0.038). Membrane fractions of monkey brains were subjected to immunoblotting, and the band density was quantitated by densitometric scanning and normalized to the corresponding β-actin density (Fig. 4). No significant difference in the relative actin-normalized density of the bands for PS1, PS2, nicastrin, APH-1a or PEN-2 was observed between young and aged brains ($p > 0.05$, Student's *t*-test).

Discussion

Our results indicate that Aβ accumulation in brain tissue increases with age in cynomolgus monkeys. Levels of accumulated Aβ in aged brains were higher in the insoluble fraction than in the soluble fraction, and Aβ42 is the primary species of Aβ deposited in the brain. These results are in good accordance with previous biochemical studies using human autopsy tissues (Funato et al. 1998, Morishima-Kawashima *et al.* 2000), and suggest that the cynomolgus brain serves as a useful model for Aβ deposition in the human brain. In addition, our results show that γ-secretase is modified in an age-dependent manner to increase relative Aβ42 production and that this modulation is significantly associated with brain Aβ accumulation.

Accumulation of Aβ42 first occurs at the age of about 20 years in cynomolgus brains in this study and at approximately 50 years of age in human brains (Morishima-Kawashima et al. 2000). In addition, neocortical Aβ deposits were observed in dogs, common marmosets and mouse lemurs as young as 8, 7 and 5.5 years of age, respectively (Uchida *et al.* 1991, Mestre-Frances *et al.* 2000, Geula *et al.* 2002). These findings suggest that there is an allometric difference in development of Aβ depositions between mammalian species. Onset of Aβ accumulation is roughly proportional to the maximal species lifespan. Maximum lifespan is considered an important species characteristic of the aging process, although the mechanisms that contribute to the aging process remain unclear (de Magalhaes *et al.* 2007). This allometric relation suggests that the molecular mechanisms underlying the aging process are causatively related to the development of Aβ deposition in the brain.

In the present study, more than 80% of monkeys over 20 years old showed Aβ42 accumulation. By contrast, Aβ42 accumulation is only observed in approximately half of human individuals over 50 years old (Funato et al. 1998, Morishima-Kawashima et al. 2000). This difference could be explained by the fact that cynomolgus apolipoprotein E (apoE) is homologous to a human apoE4 isoform that contains an arginine at residue 112 and is associated with the high incidence of AD (Marotti *et al.* 1989). ApoE isoforms differentially affect Aβ aggregation and clearance (Kim *et al.* 2009). In the human population, possession of

apoE4 alleles confers accelerated onset of cerebral Aβ deposition in a gene dose-dependent manner (Morris *et al.* 2010). Approximately 90% of apoE4 carriers over the age of 50 years had biochemically-detectable accumulation of Aβ42, whereas only 33% of the non-carriers showed Aβ accumulation (Morishima-Kawashima et al. 2000).

To date, the molecular mechanisms underlying Aβ accumulation in the brains of aged subjects and sporadic AD patients are not fully understood. Enhanced Aβ generation caused by increased activity of β-secretase, and reduced Aβ degradation caused by diminished expression of neprilysin and insulin-degrading enzyme, are proposed as candidates (Fukumoto *et al.* 2004, Caccamo *et al.* 2005). Recent studies examining γ-secretase cleavage products from non-amyloidgenic substrates such as amyloid precursor-like protein 1 and alcadein-α in the cerebrospinal fluid reveal a significantly increased rate of γ-secretase misprocessing in sporadic AD patients, which leads to a relative increase in the ratio of Aβ42 generation (Yanagida *et al.* 2009, Hata *et al.* 2011). A relative increase in Aβ42 generation by modulated γ-secretase activity is considered critical for Aβ deposition (Borchelt *et al.* 1997). However, a fundamental question that remains unanswered is whether γ-secretase activity can be sustainably modified by acquired, non-genetic causes *in vivo*. Placanica et al. (Placanica *et al.* 2009b) reported that the γ42/γ40 ratio was increased in aged mouse brains, but they did not observe spontaneous Aβ deposition. The present results

1 further support the possibility of age-dependent, acquired modulation of γ-secretase activity. Thus, the
2 misprocessing of APP by modulated γ-secretase activity might contribute to age-related Aβ deposition and
3 development of sporadic AD. This further suggests that to reverse the age-related modulation of γ-secretase
4 activity would be a reasonable therapeutic strategy for the treatment of early stage AD.

5 A consecutive-cleavage mechanism has been proposed for γ-secretase processing of APP
6 (Qi-Takahara *et al.* 2005, Takami *et al.* 2009). Familial AD-causing presenilin mutations alter the cleavage
7 efficiency at multiple sites depending on the mutation loci, which eventually results in an increase in the
8 γ42/γ40 ratio but does not always enhance the absolute production of Aβ42 (Qi-Takahara et al. 2005,
9 Bentahir et al. 2006). Besides genetic mutations of APP or presenilins, the mechanisms underlying alteration
10 of the γ42/γ40 ratio remain poorly understood. Artificial N-terminal elongation of PEN-2 or allosteric effects
11 of γ-secretase modulators cause a relative increase of Aβ42 production through a structural change of the
12 catalytic pore (Isoo *et al.* 2007). Altered composition of the γ-secretase complex is also known to affect
13 the γ42/γ40 ratio (Placanica *et al.* 2009a, Serneels *et al.* 2009). Our results indicate that a decrease in γ40
14 contributes to the age-related increase in the γ42/γ40 ratio, but its mechanism remains undetermined. An
15 important future issue will be to identify the molecular basis for the age-related modification of γ-secretase
16 activity.

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Figure legends

Figure 1

Aβ accumulation in the temporal cortex of the cynomolgus monkey. (a) Representative immunostaining for total Aβ, Aβ40 and Aβ42. Serial sections of the temporal cortices of 25-year-old (upper images) and 30-year-old (lower images) monkeys were stained for an antibody against the N-terminus of Aβ (82E1), the C-terminus of Aβ40 or the C-terminus of Aβ42. The relationship between age and the logarithm of Aβ40 (b) and Aβ42 level (c). (d) The relation between the logarithms of Aβ40 and Aβ42 levels.

Figure 2

Aβ levels in the soluble and insoluble fractions of brain tissue. (a–d) The relation between age and the level of insoluble Aβ40 (a), insoluble Aβ42 (b), soluble Aβ40 (c) and soluble Aβ42 (d). (e–g) The relation between the logarithm of insoluble Aβ42 level and the level of soluble Aβ40 (e) and Aβ42 (f). The relation between the logarithm of insoluble Aβ40 level and the level of soluble Aβ40 (g) and Aβ42 (h).

Figure 3

γ-Secretase activity in temporal cortex tissues. (a–b) The relation between age and γ40 (a) and γ42 (b) activity in all monkeys. (c–d) The relationship between age and γ40 activity in female (c) and male (d) monkeys. (e–f) The relation between age (e) and the logarithm of total Aβ42 level (f) and the ratio of γ42 to γ40.

Figure 4

Immunoblots for PS1, PS2, nicastrin, APH-1a and PEN-2 in brains of young and aged monkeys. The mean ages of young and aged monkeys were 5.5 ± 1.5 and 33.7 ± 2.4 years, respectively. The blot with anti-β-actin antibody served as a loading control. The band density was quantitated by densitometric scanning and normalized to the corresponding β-actin density. The graph shows the percentage of actin-normalized band density (the mean + SD) for each indicated protein in aged brains relative to the mean actin-normalized band density obtained for young brains.

Figure 1

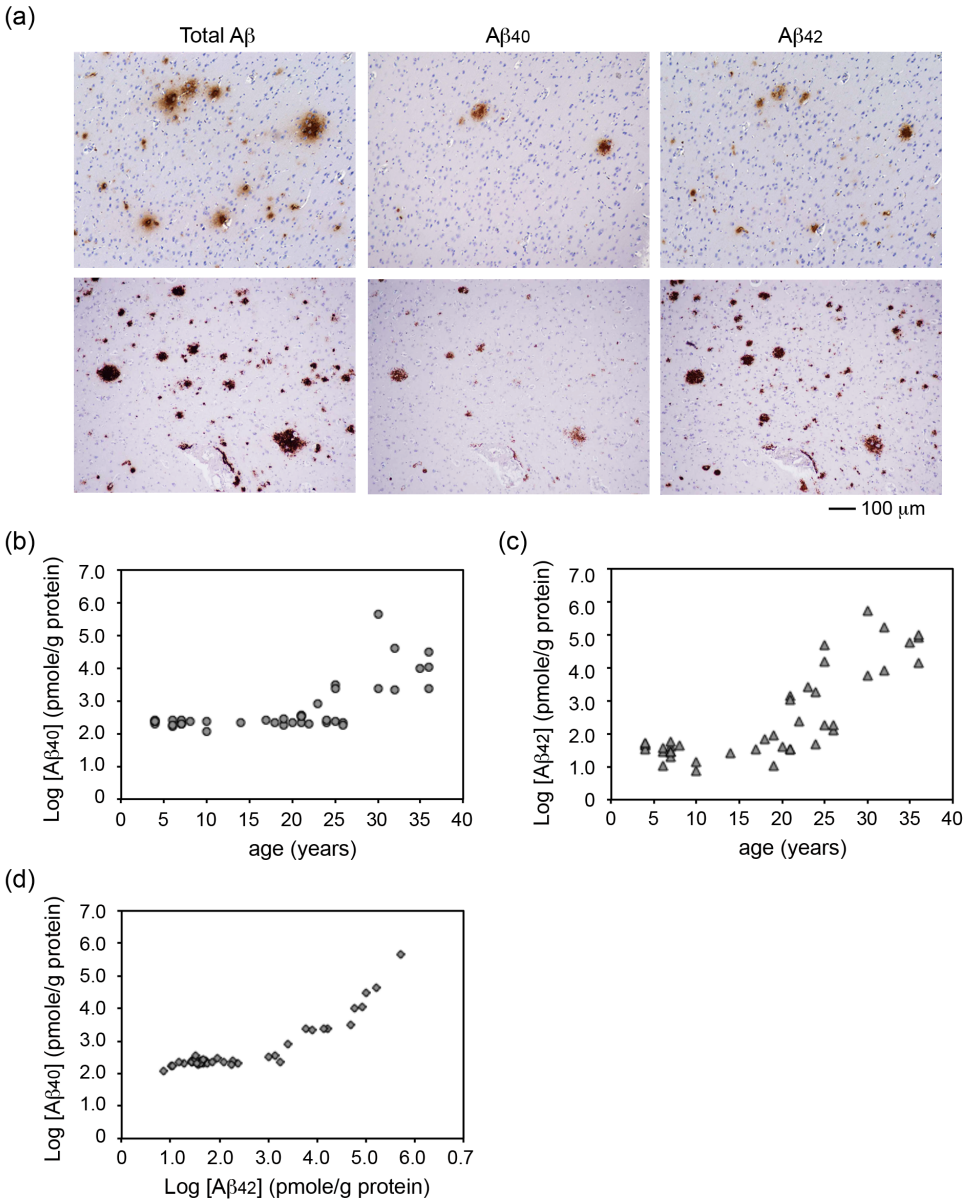

(a)
Total Aβ
Aβ40
Aβ42
100 μm
(b)
Log [Aβ40] (pmole/g protein)
age (years)
(c)
Log [Aβ42] (pmole/g protein)
age (years)
(d)
Log [Aβ40] (pmole/g protein)
Log [Aβ42] (pmole/g protein)

Figure 2

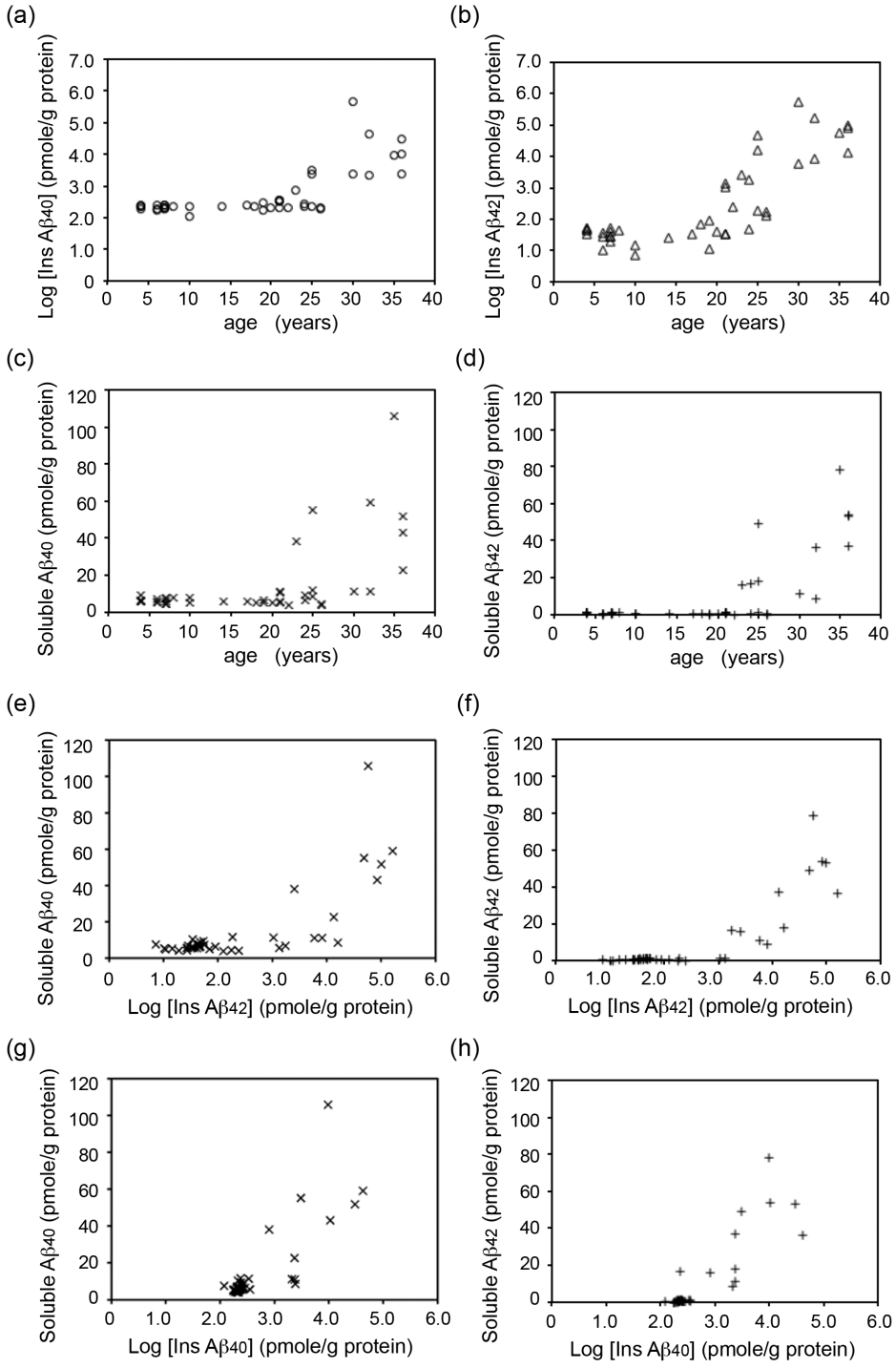

(a)
Log [Ins Aβ40] (pmole/g protein)
age (years)
(b)
Log [Ins Aβ42] (pmole/g protein)
age (years)
(c)
Soluble Aβ40 (pmole/g protein)
age (years)
(d)
Soluble Aβ42 (pmole/g protein)
age (years)
(e)
Soluble Aβ40 (pmole/g protein)
Log [Ins Aβ42] (pmole/g protein)
(f)
Soluble Aβ42 (pmole/g protein)
Log [Ins Aβ42] (pmole/g protein)
(g)
Soluble Aβ40 (pmole/g protein)
Log [Ins Aβ40] (pmole/g protein)
(h)
Soluble Aβ42 (pmole/g protein)
Log [Ins Aβ40] (pmole/g protein)

Figure 3

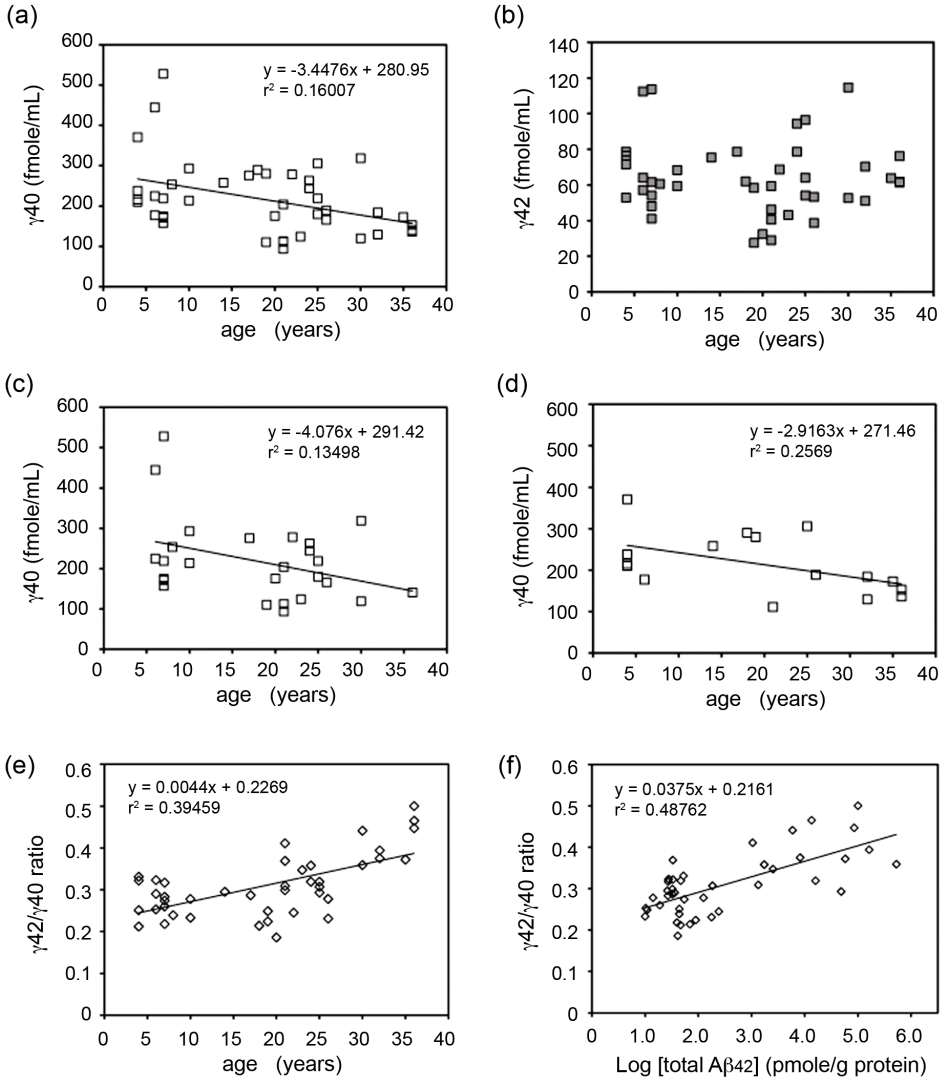
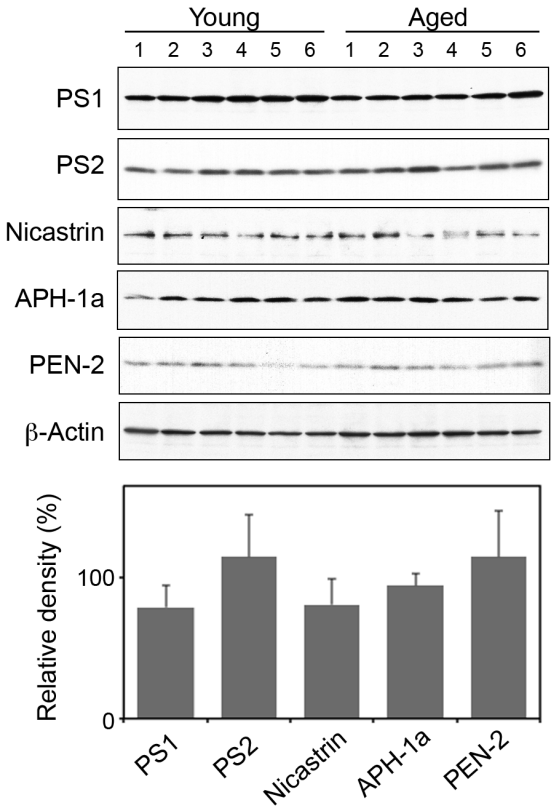

(a)
y = -3.4476x + 280.95
r² = 0.16007
γ40 (fmole/mL)
age (years)
(b)
γ42 (fmole/mL)
age (years)
(c)
y = -4.076x + 291.42
r² = 0.13498
γ40 (fmole/mL)
age (years)
(d)
y = -2.9163x + 271.46
r² = 0.2569
γ40 (fmole/mL)
age (years)
(e)
y = 0.0044x + 0.2269
r² = 0.39459
γ42/γ40 ratio
age (years)
(f)
y = 0.0375x + 0.2161
r² = 0.48762
γ42/γ40 ratio
Log [total Aβ42] (pmole/g protein)

Figure 4


Young
Aged
1 2 3 4 5 6 1 2 3 4 5 6
PS1
PS2
Nicastrin
APH-1a
PEN-2
β-Actin
Relative density (%)
100
0
PS1
PS2
Nicastrin
APH-1a
PEN-2