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**Contributors**

R. Hashimoto supervised the entire project, collected the data, wrote the manuscript, was critically involved in the design, analysis and interpretation of the data and was responsible for performing the literature review. K. Ohi was critically involved in the collection and analysis of the data, and contributed to the editing of the final manuscript and contributed intellectually to the interpretation of the data. Y. Yasuda, T. Yoshida, H. Takahashi, N. Iike, M. Fukumoto, H. Takamura, M. Iwase, K. Kamino, R. Ishii, H. Kazui, R. Sekiyama, Y. Kitamura, M. Azechi, K. Ikezawa, R. Kurimoto, E. Kamagata, H. Tanimukai, S. Tagami, T. Morihara, M. Ogasawara, M. Okochi, H. Tokunaga, S. Numata, M. Ikeda, T. Ohnuma, T. Fukunaga, T. Tanaka, T. Kudo, S. Ueno, H. Arai, T. Ohmori, N. Iwata, N. Ozaki and M. Takeda were heavily involved in the collection of the majority of the data and contributed intellectually to the interpretation of the data. All authors contributed to and have approved the final manuscript.

**Conflict of interest**

All authors declare that they have no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.schres.2009.01.019.

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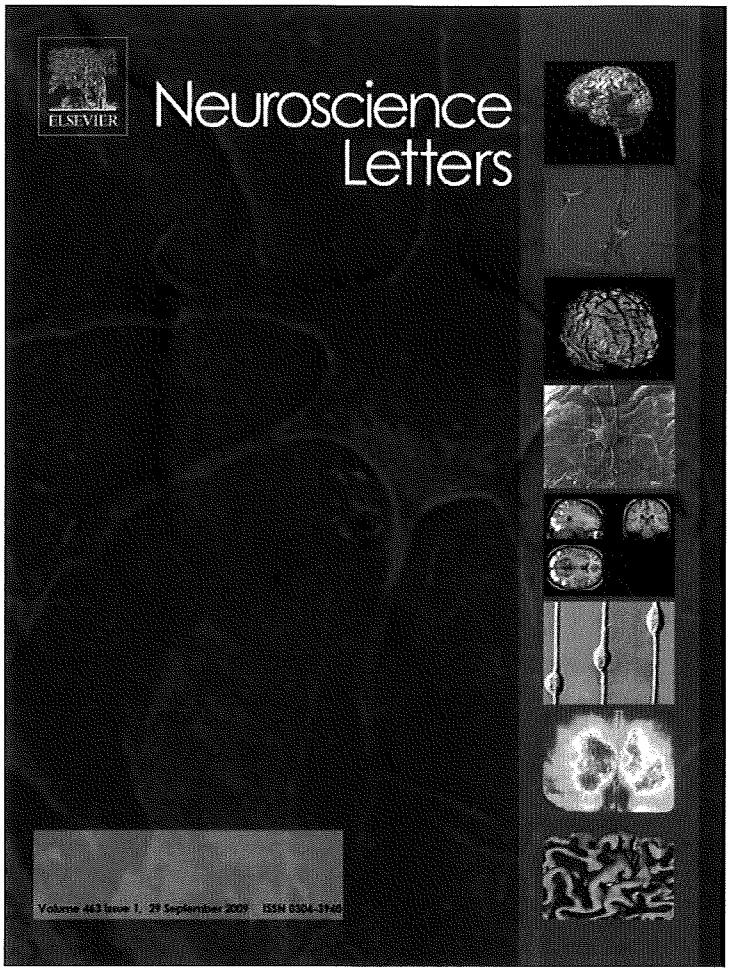

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# No association between the *Bcl2-interacting killer* (*BIK*) gene and schizophrenia

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ABSTRACT

The *Bcl2-interacting killer* (*BIK*) gene interacts with cellular and viral survival-promoting proteins, such as Bcl-2, to enhance apoptosis. The BIK protein promotes cell death in a manner analogous to Bcl-2-related death-promoting proteins, Bax and Bak. There have been lower Bcl-2 levels and increased Bax/Bcl-2 ratio in the temporal cortex of patients with schizophrenia compared with those in controls. Because the death-promoting activity of BIK was suppressed in the presence of the cellular and viral survival-promoting proteins, the BIK protein is suggested as a likely target for antiapoptotic proteins. The purpose of this study is to investigate the association between genetic variants in the *BIK* gene and schizophrenia in a large Japanese population (1181 patients with schizophrenia and 1243 healthy controls). We found nominal evidence for association of alleles, rs926328 ($\chi^2 = 4.44$, $p = 0.035$, odds ratio = 1.13) and rs2235316 ($\chi^2 = 4.41$, $p = 0.036$, odds ratio = 1.13), with schizophrenia. However, these associations were no longer positive after correction for multiple testing (rs926328: corrected $p = 0.105$, rs2235316: corrected $p = 0.108$). We conclude that *BIK* might not play a major role in the susceptibility of schizophrenia in Japanese population.



Schizophrenia (MIM 181500) is a common complex psychiatric disease and is generally considered as a neurodevelopmental disorder. The lifetime morbidity rate is 0.5–1.0% across distinct populations. Family, twin, and adoption studies of schizophrenia have indicated that there are strong genetic factors with an estimated heritability of approximately 80% [5,20]. Regions on a number of chromosomes (e.g., 1, 6, 8, 10, 13, and 22) have been implicated as sites of potential vulnerability genes [17]. For example, 22q11–13 has been shown as a suggestive region in a number of linkage studies.

Apoptosis, a form of programmed cell death, is regulated by a complex cascade of pro- and anti-apoptotic members of the Bcl-2 family proteins. The ratio of pro-apoptotic (e.g. Bax, Bad) to anti-apoptotic (e.g. Bcl-2, Bcl-$X_L$) protein levels is a key determinant in regulating cytochrome *c* release and subsequent caspase activation, leading to rapid neuronal death [16,21]. Postmortem brain studies showed that markers of apoptosis, levels of apoptotic regulatory proteins and DNA fragmentation patterns, were altered in schizophrenia [1,2,12,13]. The Bcl-2 levels are 30% lower in temporal cortex in schizophrenia compared with controls [12]. There is a 50% increase in the Bax/Bcl-2 ratio in the temporal cortex of patients with schizophrenia compared to matched controls [13]. The cortical neuropathology in schizophrenia such as synaptic deficits and reduced neuropil without overall neuronal loss, and limited and often layer-specific reductions of neurons appears to be characterized by non-lethal and localized apoptotic activity at the level of synapses and terminal neuritis in schizophrenia [1,2,8,13]. Neuroimaging studies suggest that a progressive loss of cortical gray matter occurs in the early stage of the clinical course of schizophrenia [3,7,19]. Although the mechanisms underlying these data are

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**Table 1**
Genotype and allele distributions for SNPs in the *BIK* gene between patients with schizophrenia and controls.

| Marker | | | | SCZ | | | CON | | Genotypic *p*-value (*df* = 2) | SCZ | CON | Allelic *p*-value (*df* = 1) | OR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| dbSNP IDs | Position[a] | M/m | Gene | M/M | M/m | m/m | M/M | M/m | m/m | MAF | | | |
| rs926328 | 22896778 | T/A | 5′ | 0.29 | 0.50 | 0.21 | 0.26 | 0.51 | 0.24 | 0.10 | 0.46 | 0.49 | 0.035 | 0.89 |
| rs4988372 | 22897148 | C/T | 5′ | 0.89 | 0.10 | 0.005 | 0.91 | 0.09 | 0.003 | 0.50 | 0.06 | 0.05 | 0.25 | 1.16 |
| rs4988374 | 22897266 | C/A | 5′ | 0.89 | 0.10 | 0.005 | 0.91 | 0.09 | 0.003 | 0.45 | 0.06 | 0.05 | 0.21 | 1.18 |
| rs2235316 | 22914504 | A/C | Intron 3 | 0.26 | 0.50 | 0.24 | 0.29 | 0.49 | 0.22 | 0.10 | 0.49 | 0.46 | 0.036 | 1.13 |

SCZ, patients with schizophrenia; CON, healthy controls; m, minor allele; M, major allele; MAF, minor allele frequency; OR, odds ratio.
[a] db SNP build 129.

unknown, evidence for progressive clinical deterioration and subtle neurostructural changes following the onset of psychosis has led to the hypothesis that the increased apoptotic vulnerability may contribute to the pathophysiology of schizophrenia.

*BIK* (*Bcl2-interacting killer*) gene (MIM 603392) mapped on chromosome 22q13, which has a modest linkage to schizophrenia [6]. The gene contains 5 exons and spans approximately 19 kb [22]. *BCL-2* gene has been shown to enhance the survival of a variety of cell types exposed to diverse cell death-inducing stimuli. Bcl-2-related proteins either promote cell survival or accelerate cell death. The protein encoded by the *BIK* gene is known to interact with the cellular survival-promoting proteins Bcl-2 and Bcl-$X_L$, as well as with the viral survival-promoting proteins Epstein–Barr virus (EBV) BHRF1 and adenovirus E1B 19-kD to enhance apoptosis [4,9]. The BIK promotes cell death similar to the Bcl-2-related death-promoting proteins, Bax and Bak. Because the death-promoting activity of BIK was suppressed by coexpression of Bcl-2, Bcl-$X_L$, EBV BHRF1, and E1B 19-kD, the BIK protein might be associated with anti-apoptotic proteins. In this study, we investigated whether the *BIK* gene is associated with schizophrenia in a large Japanese population.

The subjects consisted of 1181 patients with schizophrenia [51.1% males (604/577), mean age ± SD; 46.1 ± 14.9 years] and 1243 healthy controls [46.5% male (578/665), mean age ± SD; 38.6 ± 15.7 years]. The sex ratio and the mean age differed significantly between groups (sex ratio; $\chi^2 = 5.2$, $p = 0.022$, mean age; $z = -13.0$, $p < 0.001$). All the subjects were biologically unrelated Japanese. Patients were recruited at the National Center Hospital of Neurology and Psychiatry, Showa University School of Medicine, Fujita Health University School of Medicine and Osaka University Graduate School of Medicine. Cases were recruited from both outpatients and inpatients at the hospitals. Each schizophrenic research subject had been diagnosed by at least two trained psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria based on unstructured clinical interviews. Controls, including the hospital and institutional staffs, were recruited through local advertisements. Psychiatrically healthy controls were evaluated using unstructured interviews to exclude individuals who had current or past contact with psychiatric services. We did not assess the controls for the family history of psychiatric disorders, such as schizophrenia, bipolar disorder, or major depressive disorder. All experiments on human subjects were conducted in accordance with the Declaration of Helsinki and all procedures were carried out with the adequate understanding and written consent of the subjects.

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to standard procedures. Four single nucleotide polymorphisms (SNPs) in the *BIK* gene were selected from the public database (HAPMAP: http://www.hapmap.org/index.html.ja) in order to cover the *BIK* gene (4.75 kb per SNP). The four SNPs in the *BIK* gene (rs926328, rs4988372, rs4988374 and rs2235316) were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay (Applied Biosystems, Foster City, California, USA), as described previously [10,11,15]. Primers and probes for detection of the SNPs are available upon request.

Statistical analyses were performed using SNPAlyze V5.1.1 Pro software (DYNACOM, Yokohama, Japan) and SPSS 16.0J software (SPSS Japan Inc., Tokyo, Japan). Differences in clinical characteristics between patients and controls or between genotype groups were analyzed using $\chi^2$ tests for categorical variables and the Mann–Whitney *U*-test for continuous variables. A deviation from Hardy–Weinberg equilibrium (HWE) was tested separately in cases and controls by using the $\chi^2$ tests for goodness of fit. The allelic and genotypic distributions of the *BIK* polymorphisms between patients and controls were analyzed using $\chi^2$ tests. The number of effective independent SNPs assayed was estimated by the spec-

**Table 2**
Marker-to-marker linkage disequilibrium for all the combinations of the four SNPs in the *BIK* gene.

| | rs926328 | rs4988372 | rs4988374 | rs2235316 |
|---|---|---|---|---|
| rs926328 | – | 1.00 | 1.00 | 0.36 |
| rs4988372 | 1.00 | – | 1.00 | 0.88 |
| rs4988374 | 1.00 | 1.00 | – | 0.86 |
| rs2235316 | 0.39 | 0.91 | 0.91 | – |

For each pair of markers, the standardized $D'$ in controls is shown below the diagonal, and the standardized $D'$ in patients with schizophrenia is shown above the diagonal.

tral decomposition method of Nyholt using SNPSpD software [14]. The pairwise linkage disequilibrium (LD) analyses, expressed by $D'$, were applied to detect the intermarker relationship in each group using the SNPAlyze V5.1.1 Pro software. We performed power calculations using the Power Calculator for Two Stage Association Studies (http://www.sph.umich.edu/csg/abecasis/CaTS/ [18]). Power (>0.80) was calculated under prevalence of 0.01, several allele frequencies in patients (rs926328; 0.46, rs4988372; 0.06, rs4988374; 0.06, or rs2235316; 0.46) and an alpha level of 0.05 using a multiplicative model, assuming varying degrees of the odds ratio. All $p$-values reported are two tailed. Statistical significance was defined as $p < 0.05$.

The genotype and allele frequencies of four SNPs located in the *BIK* gene and the flanking regions are summarized in Table 1. Genotyping completeness ranged from 96.3% (rs2235316) to 99.3% (rs4988372). The genotype distributions of all examined SNPs in the *BIK* gene were in HWE for both controls and patients with schizophrenia ($p > 0.3$). We found no genotypic association between the four SNPs in the *BIK* gene and schizophrenia (Table 1). On the other hand, there were significant differences in allele frequencies of rs926328 and rs2235316 (Table 1). The major T allele of the rs926328 and the minor C allele of the rs2235316 in the *BIK* gene were in excess in patients with schizophrenia compared with controls (rs926328; $\chi^2 = 4.44$, $p = 0.035$, odds ratio = 1.13, 95% confidential interval 1.01–1.27, rs2235316; $\chi^2 = 4.41$, $p = 0.036$, odds ratio = 1.13, 95% confidential interval 1.01–1.27; Table 1). However, these associations did not survive after SNPSpD correction for multiple testing in this gene (the effective number of independent marker loci: 3.0; rs926328; corrected $p = 0.105$, rs2235316; corrected $p = 0.108$). There was no allelic association with schizophrenia for the other two SNPs, rs4988372 and rs4988374. The LD relationships between markers were shown in Table 2. The LD pattern observed in our patients was nearly identical to those in our controls. The strong LD patterns among four SNPs were observed in both groups ($D' > 0.8$), except for the weak LD between rs926328 and rs2235316 in both groups ($D' < 0.5$).

Nominal associations between the genetic variants, rs926328 and rs2235316, and schizophrenia in this study were no longer positive after correction for multiple testing. Power analysis showed that our subjects had sufficient power (>0.80) to detect an effect of the odds ratio (1.18 or more) for rs926328 or rs2235316. For other two SNPs, rs4988372 and rs4988374, our sample size had power (>0.80) to detect an effect of the odds ratio (1.38 or more). As the odds ratios of the rs926328 and rs2235316 were 1.13, our sample size was not enough to detect such a small contribution to risk for schizophrenia. This could lead to the type II error. There were other limitations in our study. Control samples were not matched by age and sex with the patient population or were not represent general population as they included substantial portion of hospital and institutional staffs. Although the psychiatric phenotypes of the controls were screened by the unstructured interview, we did not assess the family history of controls subjects. Thus, control subjects might carry the risk allele from affected parents. Careful interpretation of our results due to population stratification would be needed in our case control designed study, despite the precaution of ethnic matching of this study. Therefore, it is necessary to carry out further investigations to confirm our findings in other samples with age and sex matched case-control subjects and with much larger sample size.

In conclusion, we have examined a possible association between the *BIK* gene and schizophrenia for the first time and have not found the association with schizophrenia in the Japanese population. Two SNPs gave nominal evidence for association and this association did not survive after correction for multiple testing. *BIK* is not likely to be a major susceptibility gene for schizophrenia in this Japanese population. The diagnosis of schizophrenia itself is too non-specific and that the *BIK* might be more related to differences in patients with and without specific deficits in basic neurophysiological process such as sensory gating, executive function, etc. Further studies are needed to investigate the contribution of pro- or anti-apoptotic genes other than *BIK* in schizophrenia.

## Acknowledgements

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# The 28-amino acid form of an APLP1-derived Aβ-like peptide is a surrogate marker for Aβ42 production in the central nervous system

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Surrogate markers for the Alzheimer disease (AD)-associated 42-amino acid form of amyloid-β (Aβ42) have been sought because they may aid in the diagnosis of AD and for clarification of disease pathogenesis. Here, we demonstrate that human cerebrospinal fluid (CSF) contains three APLP1-derived Aβ-like peptides (APL1β) that are generated by β- and γ-cleavages at a concentration of ~4.5 nM. These novel peptides, APL1β25, APL1β27 and APL1β28, were not deposited in AD brains. Interestingly, most γ-secretase modulators (GSMs) and familial AD-associated presenilin1 mutants that up-regulate the relative production of Aβ42 cause a parallel increase in the production of APL1β28 in cultured cells. Moreover, in CSF from patients with pathological mutations in *presenilin1* gene, the relative APL1β28 levels are higher than in non-AD controls, while the relative Aβ42 levels are unchanged or lower. Most strikingly, the relative APL1β28 levels are higher in CSF from sporadic AD patients (regardless of whether they are at mild cognitive impairment or AD stage), than those of non-AD controls. Based on these results, we propose the relative level of APL1β28 in the CSF as a candidate surrogate marker for the relative level of Aβ42 production in the brain.

## INTRODUCTION

A key feature of the pathology of AD is the accumulation of amyloid-β peptides (Aβ) in senile plaques (Masters et al, 1985; Selkoe, 2001). Aβ is produced *via* endoproteolysis by BACE, which cleaves βAPP at the extracellular domain (Hussain et al, 1999; Sinha et al, 1999; Vassar et al, 1999; Yan et al, 1999), and by the presenilin (PS)–γ-secretase complex (Francis et al, 2002;

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Yu et al, 2000), which cleaves βAPP in the transmembrane domain (TM) (De Strooper, 2003; Edbauer et al, 2003; Kimberly et al, 2003; Takasugi et al, 2003). To date, no Aβ-like TM-containing peptides from other type-1 TM proteins have been found in brain. However, because *in vitro* studies indicate that Aβ-like peptides derived from Notch-1, CD44, βAPP like protein 1/2 (APLP1/2), alcadein, β-subunits of voltage-gated sodium channels and interleukin-1 receptor II are secreted by cultured cells (Araki et al, 2004; Eggert et al, 2004; Kuhn et al, 2007; Lammich et al, 2002; Okochi et al, 2002, 2006; Wong et al, 2005), we suspected that Aβ-like peptides may exist *in vivo*.

Aβ42 is a major constituent of senile plaques and is thought to induce the pathological process of AD (Selkoe, 2001). Thus the level of Aβ42 production in the brain, especially relative to total Aβ production, is a potential biomarker of the pathological process in AD. However, in patients with AD, the relative ratio of Aβ42 to total Aβ in CSF is lower (Andreasen et al, 1999; Jensen et al, 1999; Motter et al, 1995), probably because Aβ42 in the brain is being cleared more rapidly from the soluble pool by an enhanced rate of deposition/aggregation. To date, surrogate markers for estimating Aβ42 generation in the brain have not been identified. Such surrogate markers might reveal an increased ratio of Aβ42 production and is also associated with the pathology of sporadic AD cases.

In this study, we demonstrate that human CSF contains novel APLP1-derived APL1β species. Our data indicate that brain APL1β28 levels are a surrogate marker for the brain Aβ42 production, as the relative ratio of APL1β28 was up-regulated in CSF samples from patients with a variety of familial AD mutations and sporadic AD.

## RESULTS

### Identification of novel Aβ-like peptides (APL1β) derived from APLP1 in human CSF

APLP1 and APLP2 are similar to βAPP in the primary sequence and homologous in function (Coulson et al, 2000). To find an *in vivo* Aβ-like peptide, we focused on APLP1 and raised antibodies against the juxtamembrane domain (IQRDELAPAGTGVSRE for OA601 and DELAPAGTGVSRE for OA663). Human CSF was obtained by lumbar puncture from non-demented patients, and proteins were immunoprecipitated using these antibodies or anti-Aβ antibody 4G8. The molecular masses of the precipitated proteins were analysed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectroscopy (MS). Experiments using OA601 or OA663 detected an identical set of three peptides of 2,329, 2,473and

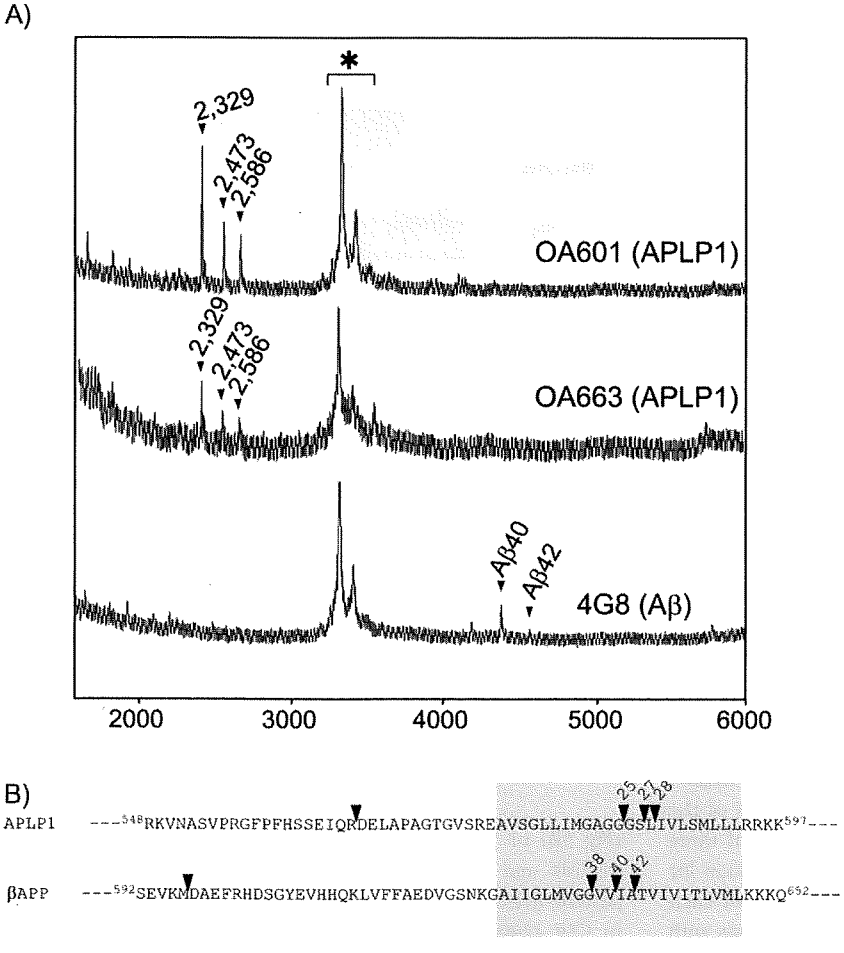


**Figure 1. MALDI-TOF MS analysis of APLP1 peptides in human CSF.**

**A.** Determination of the molecular masses of APLP1 peptides in human CSF. Human CSF (300 μl) was immunoprecipitated with the indicated antibodies and analysed by MALDI-TOF MS. Numbers shown on top of the peaks are molecular masses. Asterisks (*) indicate nonspecific peaks.

**B.** A diagram of the APL1β and Aβ domains in APLP1 and βAPP sequences, respectively. Arrowheads and the grey boxes indicate cleavage sites and the deduced TM, respectively. The numbers above the arrowheads indicate the number of amino acid residues in each fragment.

  

2,586 Da (Fig 1A). Under the same conditions, the Aβ species were recognized by 4G8 (Fig 1A). On the basis of the molecular weights and the epitopes recognized by the antibodies, we presumed the amino acid sequences of the set of APLP1 peptides. These peptides were named APL1β25 (calculated MW 2,327.2 Da), APL1β27 (calculated MW 2,471.3 Da) and APL1β28 (calculated MW 2,584.3 Da) to reflect the number of amino acid residues in each peptide (see Table S1 of Supporting Information). Finally, the amino acid sequences were determined by using a liquid chromatography-tandem MS (LC/MS/MS) system (see Fig S1 of Supporting Information). Similar to Aβ, the novel brain peptide species derived from APLP1 have a juxtamembrane region at their common N-terminus and a part of the hydrophobic TM at their variable C-termini (Fig 1B).

### Sequential endoproteolytic processing by BACE and PS/γ-secretase produces the APL1β species in untransfected SH-SY5Y cells

We suspected that APL1β is generated by a similar process as Aβ. Since naïve SH-SY5Y human neuroblastoma cells were found to secrete the same APL1β species as those found in the human CSF (Fig 2A), degradation of endogenous APLP1 in the cells was then analysed by immunoprecipitation (IP)-MS analysis (Fig 2A). The cells were also radiolabelled with [$^{35}$S] methionine overnight (Fig 2B) and analysed by IP-autoradiography (Fig 2B; second and fourth panels). Both the IP-MS analysis and the pulse-chase experiments revealed that treatment with a BACE1/2 inhibitor, inhibitor IV, abolishes APL1β secretion. In addition, recombinant BACE1/2 cleaved an APLP1 peptide (Nma-EIQRDELAK(Dnp)-RR-NH2) containing the N-terminus of APL1β as well as a wild-type (wt) βAPP peptide (Nma-EV*KM*DAEFK(Dnp)-RR-$NH_2$), which contains the N-terminal sequence of Aβ (see Fig S2 of Supporting Information). These results suggest that BACE1/2 can participate in the generation of APL1β which is reminiscent of Aβ generation (Farzan et al, 2000; Hussain et al, 2000).

To determine if PS/γ-secretase is involved in the secretion of APL1β, the cells were treated with the γ-secretase inhibitors DAPT (Fig 2A and B) and L685,458 (Fig 2B). Both compounds abolished APL1β secretion and concomitantly induced intracellular accumulation of APLP1 C-terminal fragment (CTF) stubs, the substrate for γ-cleavage (Fig 2B; third and fourth panels). These results suggest that sequential endoproteolysis by BACE1/2 and PS/γ-secretase mediates APL1β generation.

### APL1β and Aβ levels are comparable in human CSF

We next determined how much APL1β is present in the CSF. To do this, an LC/MS/MS system was established to measure the level of each APL1β species. The LC/MS/MS analysis was first performed using synthetic APL1β peptides to select optimal 'daughter (or product)' ions and conditions for quantification (b2, y20 and y21 for APL1β25/28; b2, y21 and y22 for APL1β27; see Fig S3 of Supporting Information). The peak areas of the three daughter ions were measured and the average of three calculated concentrations was defined as the concentration for each APL1β species. Subsequently, various amounts of each synthetic peptide were added to the CSF (50 μl) of patients with

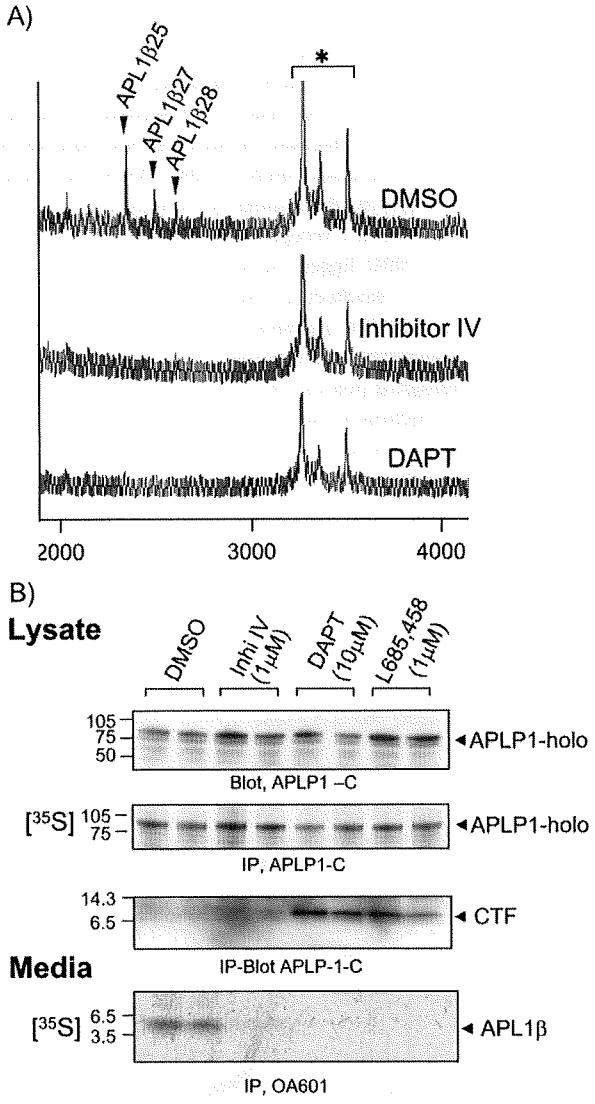


**Figure 2. APL1β secretion from naïve SH-SY5Y cells.** Inhibition of APL1β secretion from SH-SY5Y cells upon treatment with an inhibitor of BACE1/2 (1 μM inhibitor IV) or PS/γ-secretase (10 μM DAPT or 1 μM L685458).

**A.** Conditioned media were analysed by IP-MS. Asterisks (*) indicate non-specific peaks.

**B.** Cells were radiolabelled with [$^{35}$S] methionine overnight. APL1β (detected via IP-autoradiography) was detected in the conditioned medium (fourth panel). Note that [$^{35}$S] incorporation in the APL1β bands was abolished by the inhibitors. APLP1 holoprotein (detected *via* direct blotting and IP-autography; first and second panels) and APLP1-CTF stubs (detected *via* IP-blotting; third panel) were detected in the resultant cell lysates. Accumulation of APLP1-CTF stubs upon PS/γ-secretase inhibitor treatment indicates inhibition of degradation of APLP1-CTF stubs, which are the final substrate for APL1β generation following BACE cleavage. Experiments were performed in duplicate (two independent culture dishes).

(Fig 3B) or without AD (Fig 3A). The amounts of synthetic peptide and areas of the resultant daughter ions change in parallel in both the cases. Thus, the levels of each APL1β species in CSF (200 μl) were measured in this system. The APL1β

  

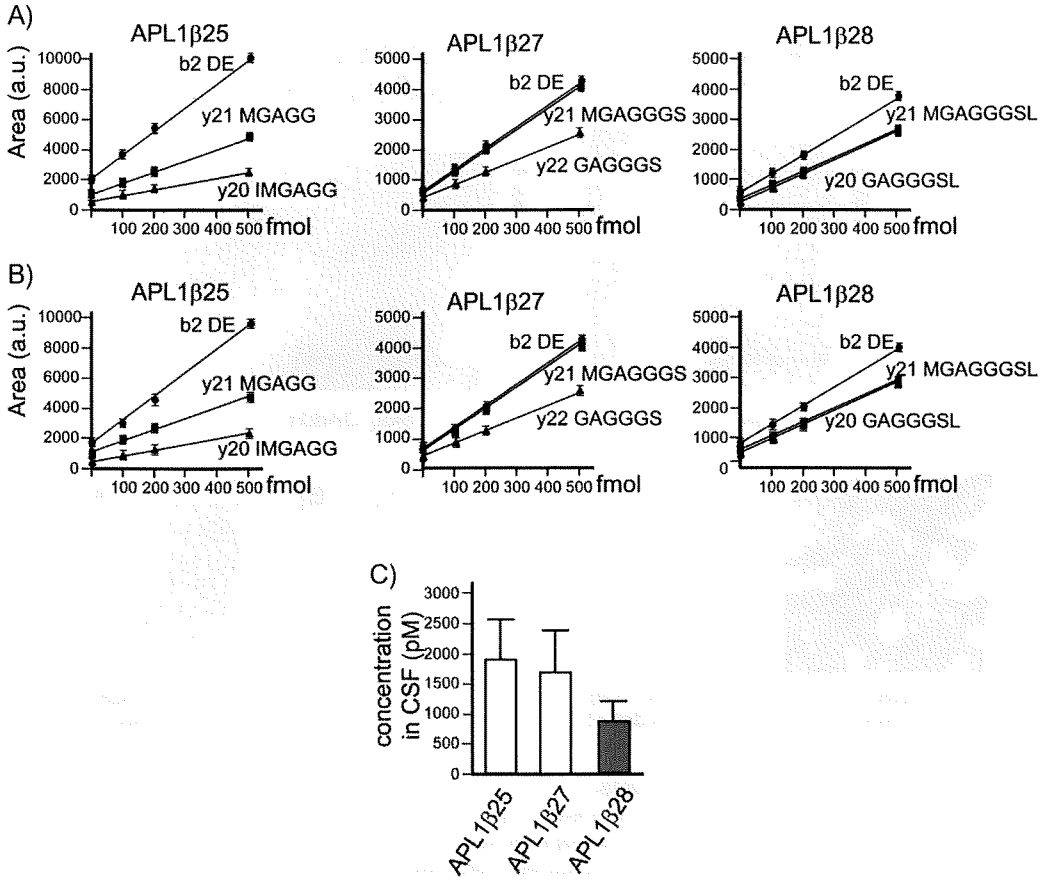


**Figure 3. Quantification of APL1β in human CSF by LC/MS/MS.**

**A.** X-axis, levels of the synthetic APL1β added to the CSF; Y-axis, areas of the indicated daughter ions. Note that none of the Y-intercepts is zero. This is due to the presence of APL1β in CSF (C100; non-AD). The experiments were performed three times and values represent mean ± SD.

**B.** The experiments were repeated using another CSF sample (C114; sporadic AD).

**C.** Comparison of the level of each APL1β species in human CSF ($n = 17$).

level in CSF from non-demented patients was 4.5 ± 1.7 nM (mean ± S.D. [$n = 17$]; see Table S2 of Supporting Information), and the concentrations of APL1β25, APL1β27 and APL1β28 were 1.9 ± 0.69, 1.7 ± 0.72 and 0.94 ± 0.39 nM, respectively (Fig 3C). Thus, considering the total Aβ level in human CSF (500 pM to 4 nM, depending on the experimental methods for the measurement) (Fukuyama et al, 2000; Ida et al, 1996; Kauwe et al, 2007; Mehta et al, 2000; Southwick et al, 1996; Wiltfang et al, 2007), the results indicate that the level of APL1β in the CSF is similar to or even higher than that of Aβ.

### APL1β is not a senile plaque component in AD

We investigated whether APL1β accumulates in senile plaques, as is the case for Aβ. The majority of Aβ in senile plaques is highly insoluble but can be obtained by extraction of the sodium dodecyl sulphate (SDS)-insoluble fraction with 70% formic acid (FA; *i.e.*, FA fraction). Thus, we analysed how much APL1β and Aβ are in the fraction of AD brain samples. The number of Aβ (including smeared bands) levels was calculated by comparison of the densitometric values of the fractions and of synthetic Aβ (see Fig 4A and Fig S4 of Supporting Information). The FA fractions from 2.5 mg of AD brain samples ($n = 2$) contained 0.40–0.80 μg of Aβ (Fig 4A). However, the FA fractions from 65 mg of the same AD brains contained less than 0.1 μg of APL1β (Fig 4B), indicating that the FA fraction of AD brains contains much less APL1β than Aβ (<1%). Immunohistochemical analysis also indicated that neither of the anti-APL1β antisera stain senile plaques in AD temporal lobe tissues (see Fig S5 of Supporting Information). To further characterize the non-aggregative nature of APL1β, we incubated APL1β *in vitro* under Aβ fibril/protofibril formation conditions, and analysed the solution by electron microscopy and size exclusion chromatography (SEC). Even though each APL1β peptide was incubated much longer than Aβ40, no APL1β fibril/protofibril formation could be detected (see Fig 4C–F and Fig S6 of Supporting Information). Collectively, the data indicate a non-amyloidogenic character of APL1β peptides.

  

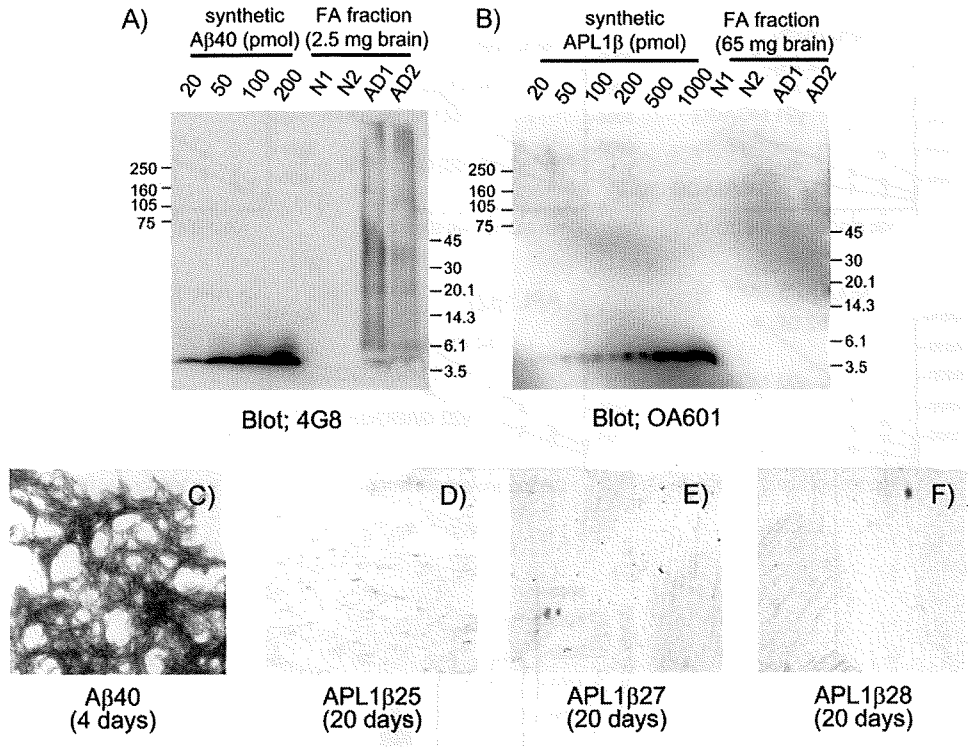


**Figure 4. Biochemical and electron microscopic analysis of APL1β.**

**A.** Aβ in the FA fraction of sporadic AD brain samples. The indicated levels of synthetic Aβ40 and Aβ42 mixtures (1:1) and brain FA fractions (extracted from 2.5 mg of non-demented [N1 and N2] or sporadic AD [AD1 and AD2] brain tissue) were subjected to SDS-PAGE and analysed by immunoblotting with monoclonal antibody 4G8. Aβ levels in each FA fraction-containing lane were calculated from the optical densities by comparison with the optical densities of synthetic peptide bands (left four lanes) as a standard.

**B.** APL1β in the FA fraction. Subsequently, the indicated levels of synthetic APL1β25, APL1β27 and APL1β28 mixtures (1:1:1) and much higher amounts of brain FA fraction (extracted from 65 mg of brain tissue) were immunoblotted with the antibody OA601. Note that OA601 detected a positive signal in the synthetic APL1β mixture but not in the FA fractions.

**C–F.** Negative staining of Aβ/APL1β peptides incubated *in vitro*. Precipitated fibrils were observed when synthetic Aβ40 was incubated for four days (C). For synthetic APL1β25, 27 and 28 peptides, no fibrils were observed (D–F, respectively).

### Some GSMs increase the relative production of APL1β28 in parallel with that of Aβ42 in cell culture

Assuming that the ratio of Aβ42 to total Aβ production in the brain increases in AD, we reasoned that a surrogate marker for Aβ42 production would be a potential biomarker for progression of AD pathology. We studied whether the levels or production of any of the APL1β species might correlate with Aβ42 levels/production. The GSM, S2474, which increases the relative Aβ42 level (Kukar et al, 2005), was added to naïve SH-SY5Y cells, and the levels of secreted APL1β and Aβ species were measured. When the concentration of S2474 in the conditioned medium was increased to 30 μM, the ratio of APL1β28 to total APL1β increased ($R^2 = 0.983$, $t = 29.0$, $p = 1.35 \times 10^{-14}$; Fig 5A). By measuring Aβ40 and Aβ42 generation in conditioned media (Fig 5B), we were able to confirm that increasing the concentration of the compound resulted in an increase in the relative Aβ42 level ($R^2 = 0.9495$, $t = 16.7$, $p = 3.93 \times 10^{-11}$; Fig 5B). Importantly, S2474 increased the ratio of APL1β28 to total APL1β (sum of APL1β25, 27 and 28) in parallel with that of Aβ42 ($R^2 = 0.9578$, $t = 18.4$, $p = 1.02 \times 10^{-11}$; Fig 5C). Similar results were obtained with fenofibrate, another GSM (see Fig S7A of Supporting Information). Thus, the results suggest that APL1β28 generation increases in parallel with Aβ42 generation under these conditions. This is reminiscent of our previous results showing that, among the Nβ species secreted from mouse Notch-1 receptor, some compounds including S2474 and fenofibrate caused parallel changes in the ratios of the longer Nβ25 form to total Nβ and of Aβ42 to total Aβ (Okochi et al, 2006).

Recently, fenofibrate and flubiprofen have been reported to modulate the γ-cleavage by binding to βAPP (Kukar et al, 2008). Although sulindac sulfide (Weggen et al, 2001) and compound-W (Okochi et al, 2006) have been shown to lower the relative Aβ42 level to total Aβ, they did not cause a decrease in the relative APL1β28 level to total APL1β (see Fig S7B–D of Supporting Information). Our results demonstrate that some GSMs, but not all, affect intramembrane proteolysis of human APLP1 as well as βAPP/mNotch-1.

  

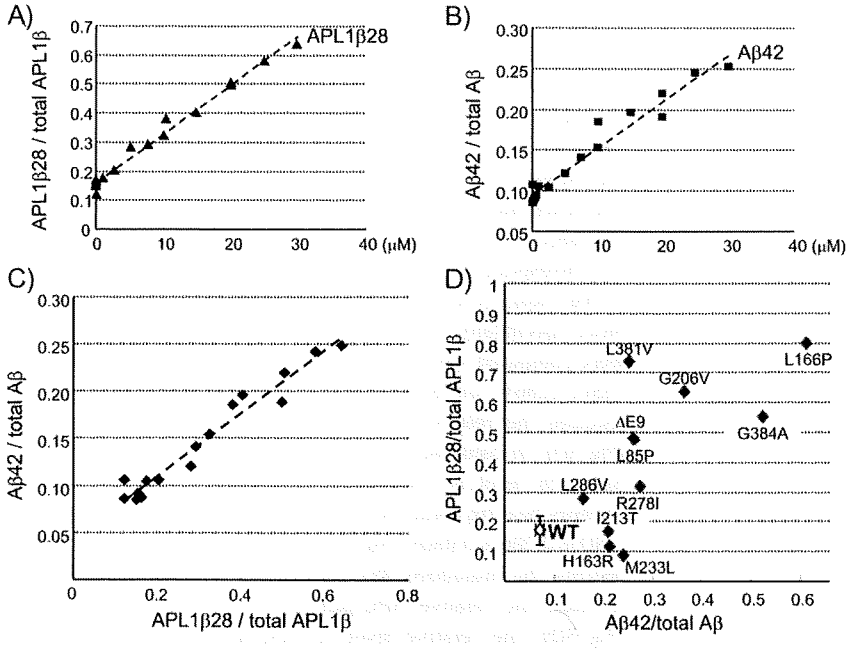


**Figure 5. Effects of GSM and PS1 familial AD mutations on the relative ratio of APL1β28 production in cell culture.** The levels of APL1β and Aβ in conditioned medium were determined using IP-LC/MS/MS or the sandwich ELISA system, respectively.

- **A.** Changes in relative APL1β28 levels after the addition of the indicated concentrations of S2474.
- **B.** Changes in relative Aβ42 levels after the S2474 addition. Note that the generation of Aβ42 increased as the dose of S2474 was increased.
- **C.** Relationship between the relative levels of APL1β28 *versus* Aβ42 upon S2474 treatment.
- **D.** Relationship between the relative levels of APL1β28 *versus* Aβ42 secreted from K293 cells stably expressing the indicated mutant forms of PS1 FAD. The values indicate means of two independent experiments without SEM.

### Several FAD-associated presinilin 1 (PS1) mutants up-regulate the relative production of APL1β28 as well as that of Aβ42 in cell culture

We also examined whether FAD mutants in PS1 can cause, in addition to their effect on Aβ42, an increase in the ratio of APL1β28 to total APL1β. Previous studies using PS1 mutant-transgenic mice report that the magnitude of the increase in the relative Aβ42 level in cultured cells reflect the magnitude of increase in the brain (Borchelt et al, 1996; Citron et al, 1997). We chose the mutants PS1 L85P, H163R, L166P, G206V, I213T, M233L, R278I, L286V, L381V, G384A and ΔE9. We prepared human embryonic kidney 293 (HEK293) cells stably expressing βAPP Swedish (sw), wt APLP1, and each of the selected PS1 FAD mutant forms. In the stable cell lines, endogenous PS proteins in PS/γ-secretase complex were successfully displaced by the exogenous mutant form (see Fig S8 of Supporting Information). Analysis of conditioned media revealed that many of the PS1 mutants increase the relative level of APL1β28 and Aβ42 in parallel (Fig 5D).

However, three of the mutant forms, PS1 H163R, I213T and M233L, were exceptions and had very minor or no effects on the rate of increase in relative APL1β28 production (wt PS background, mean ± SD = 0.163 ± 0.031) (Fig 5D). This is reminiscent of our previous finding that FAD-associated PS1 C92S does not increase the relative amount of Nβ25 generated from Notch1 (Okochi et al, 2006). Thus, there may be PS1 mutant forms that affect the interaction between βAPP and PS/γ-secretase but not the interaction between APLP1 and PS/γ-secretase.

### In CSF from patients and in cultured cells, some PS1 FAD mutations cause a parallel increase in the ratio of APL1β28 to total APL1β

As APL1β28 is not detected in insoluble AD brain fractions (Fig. 4), we examined whether the relative APL1β28 level in CSF reflects the relative production of Aβ42 in the brain. We prepared CSF from patients bearing PS1 FAD mutants (PS1 L85P, H163R, G206V, M233L, L286V and L381V) (Table 1) and from non-demented patients (see Table S2 of Supporting Information), and measured the level of each APL1β and Aβ species in the samples (Fig. 6).

**Table 1. Clinical information of the familial AD patients in this study**

| Mutation | Diagnosis | Age of onset (Year) | Age of CSF collection (Year) | Symptoms |
|---|---|---|---|---|
| PS1 L85P | Early onset AD with spastic paraplegia (variant type AD) | 26 | 27 | MMSE23/30 |
| PS1 H163R | Early onset AD with parkinsonism | 41 | 48 | MMSE3/30 |
| PS1 G206V | Early onset AD with psychosis | 37 | 38 | MMSE10/30 |
| PS1 M233L-1 | Early onset AD with parkinsonism | 41 | 48 | MMSE2/30 |
| PS1 M233L-2 | Early onset AD with spastic paraplegia | 37 | 53 | MMSE0/30, bed-ridden |
| PS1 L286V | Early onset AD | 40 | 47 | MMSE22/30, CDR1 |
| PS1 G381V | Early onset AD with spastic paraplegia (variant type AD) | 29 | 57 | MMSE0/30, bed-ridden |

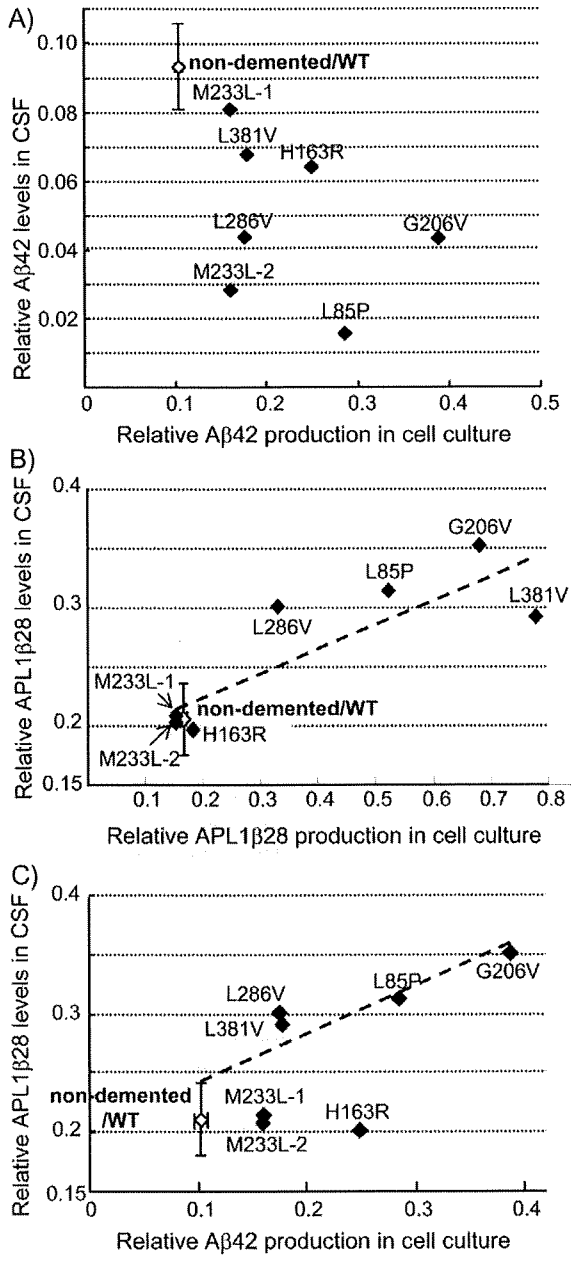


**Figure 6. Relative APL1β28 levels in CSF of PS1 FAD patients.**

**A.** Relationship between the relative ratio of Aβ42 level to total Aβ level in the CSF (for the non-demented patients with wt PS1 [$n = 17$] or patients with the indicated PS1 FAD mutations) and the relative ratio of Aβ42 production to total Aβ production in cell culture (K293 cells stably expressing wt PS1 or the indicated PS1 FAD mutants). For non-demented patients, the value is a mean, and the error bar represents the SD.

**B.** Relationship between the relative ratio of APL1β28 level to total APL1β level in the CSF and the relative ratio of APL1β28 production to total APL1β production in cell culture. Patients M233L-1 and M233L-2 are brothers.

**C.** Relationship between the relative ratio of APL1β28 level to total APL1β level in the CSF and the relative ratio of Aβ42 production to total Aβ production in cell culture.

We first calculated the relative Aβ42 levels in the CSF and then compared the values with those secreted from wt PS or the PS mutant-expressing cells (Fig 6A). As expected, although the relative levels of Aβ42 secreted from the mutant cells were elevated (wt PS background, mean ± SD = 0.104 ± 0.004), the relative levels of Aβ42 in the CSF were unchanged or decreased (non-demented patients, mean ± SD = 0.093 ± 0.010) (Fig 6A). Thus, it seems that the relative Aβ42 levels in CSF do not correspond to the relative generation of Aβ42 in FAD brains.

We, next, performed a similar study with APL1β (Fig 6B). More specifically, the relative APL1β28 levels in mutant CSF were compared with the levels secreted from mutant cells. PS1 L85P, L286V, G206V and L381V mutants were found to up-regulate the relative APL1β28 level in conditioned medium (Fig 5D). In contrast to what was found for Aβ42, the relative APL1β28 level is, indeed, up-regulated in CSF of the FAD patients bearing these mutants (non-demented patients, mean ± SD = 0.208 ± 0.030) (Fig 6B). However, in CSF of patients bearing the mutations PS1 H163R and M233L, which do not increase the relative APL1β28 level in conditioned medium (Fig 5D), the relative level of APL1β28 is not up-regulated (Fig 6B). These results suggest that, as for the PS1 FAD mutations, the relative APL1β28 levels in CSF correlate with the relative generation of APL1β28 in the brain ($R^2 = 0.74$, $t = 4.10$, $p = 0.006$; Fig 6B). Collectively, we found that PS1 FAD mutations that increase the relative levels of Aβ42 and APL1β28 in cell culture, also elevate the relative CSF levels of APL1β28 but not Aβ42 (Fig 6C). Thus, APL1β28 is a potential surrogate marker for Aβ42. That is the relative level of APL1β28 in CSF correlated with the relative generation of Aβ42 in the brain ($R^2 = 0.79$, $t = 3.31$, $p = 0.04$; Fig 6C).

### Even at the mild cognitive impairment (MCI) stage, the relative level of APL1β28 in the CSF of sporadic AD patients is higher than that of non-AD controls

Given that APL1β28 may be a non-aggregating surrogate marker for Aβ42, we examined whether the relative ratio of APL1β28 in the CSF changes in sporadic AD patients. We measured APL1β and Aβ species in CSF of patients with sporadic AD (M = 43) including those who were at the MCI stage ($n = 9$) and in demented patients without AD ($n = 35$) as well as in non-demented individuals ($n = 17$) (Supplementary Table 2). The relative ratios of APL1β28 are plotted against those of Aβ42 in Fig 7A. As clearly shown, results for many of the sporadic AD patients were located in the lower-right part (a location where APL1β28 ratio is high and Aβ42 ratio is low) of the plot. Statistically, even when the patients were at the MCI stage (mean ± SEM = 0.248 ± 0.003), the relative APL1β28 level in the CSF of sporadic AD patients (0.263 ± 0.001) was higher than that of non-demented (0.208 ± 0.002) or non-AD patients (0.212 ± 0.002) with significant differences between non-demented and sporadic AD patients ($p < 0.0001$), non-demented and MCI-stage patients ($p < 0.01$), non-AD and sporadic AD patients ($p < 0.001$), and non-AD and MCI-stage patients ($p < 0.05$), according to the Kruskal–Wallis and Wilcoxon–Mann–Whitney tests (Fig 7B). In addition, we also observed the lowered tendency of the relative Aβ42 level in the CSF of

  

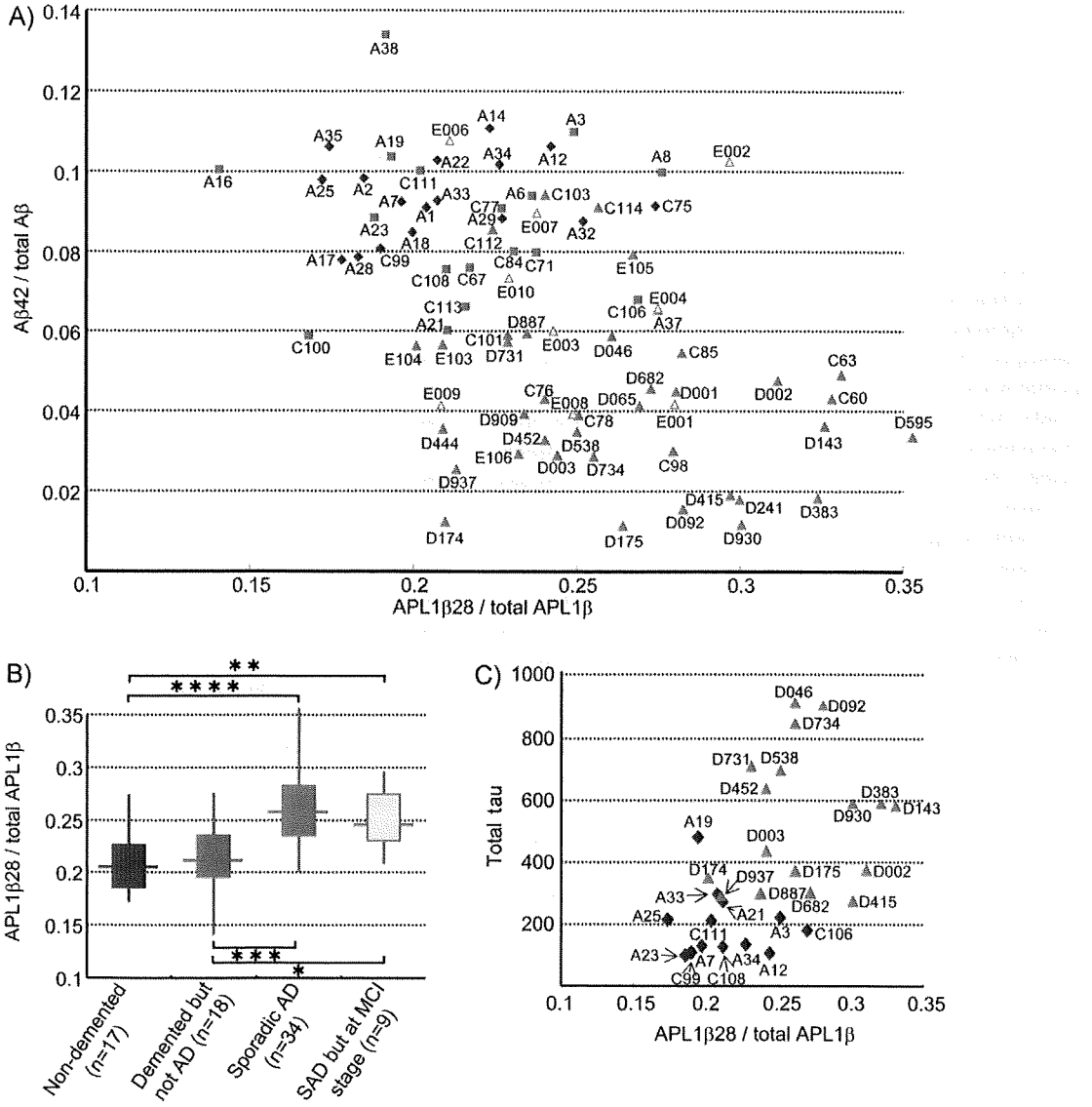


**Figure 7. Relative APL1β28 levels in the CSF of sporadic AD and other patients.**

**A.** Relationship between the APL1β28 and the Aβ42 ratio in the CSF. Non-demented, blue rhombus; non-AD, green squares; sporadic AD, red triangles; and sporadic AD at the MCI stage, yellow-filled red triangles.

**B.** Box plot of the APL1β28 ratio in the CSF of the non-demented, non-AD, sporadic AD and sporadic AD at the MCI stage. Nonparametric statistical analysis was performed ($^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$).

**C.** Relationship between the APL1β28 ratio and total tau levels in the CSF. Non-AD, blue rhombus; SAD patients, red triangles.

sporadic AD patients (Andreasen et al, 1999; Jensen et al, 1999; Motter et al, 1995). Finally, the total tau levels, another CSF biomarker of AD, were plotted against the APL1β28 ratios in the CSF of sporadic AD patients and non-demented controls (Fig 7C). Interestingly, the results for sporadic AD patients were located in the upper-right part of the plot. These results indicate that relative APL1β28 levels in CSF are useful for segregating sporadic AD patients. Additionally, no evidence was found for an altered rate of APL1β28 species degradation in CSF (Supplementary Fig 9). Therefore, the relative production of APL1β28 may be up-regulated in the brains of sporadic AD patients.

## DISCUSSION

In this study, we identified a novel APLP1-derived peptide species in human CSF. Like AD-associated Aβ, the APL1β are a group of Aβ-like peptides (Okochi et al, 2002) secreted during sequential endoproteolysis by BACE and PS/γ-secretase. Although the APL1β level in CSF is comparable to that of Aβ, we detected the APL1β species in neither senile plaques nor insoluble fractions of AD brain. In cultured cells expressing endogenous wt PS1, some GSMs caused a parallel increase in the relative levels of Aβ42 and APL1β28, the C-terminally elongated

species. Several PS1 FAD mutants also induced such effects. APL1β and Aβ peptide levels were measured in the CSF of patients with PS1 FAD pathological mutations. We found that, although the relative Aβ42 levels in CSF decreased in most of the cases, the relative APL1β28 levels in CSF and the relative APL1β28 generation by mutant-expressing cells increased in parallel. Thus, the results indicate that the level of the novel peptide APL1β28 in CSF is a candidate surrogate marker for brain Aβ42 production.

Disturbances in Aβ42 degradation/clearance are thought to play important roles in the emergence of sporadic AD pathologies (Saido and Iwata, 2006). So far, no study has reported whether there are abnormalities in Aβ42 production in sporadic AD. In this study, we found that APL1β28 and Aβ42 are secreted *in vivo* via similar processes. Thus, we investigated whether the relative level of APL1β28 in the CSF changes in patients with sporadic AD to address whether Aβ42 production changes in the brains of these patients. Strikingly, in sporadic AD, the relative APL1β28 levels in CSF were higher than that of non-AD controls. Moreover, we did not find any evidence for altered degradation of APL1β28 species in the CSF. Collectively, we suggest that (i) relative Aβ42 production in the brain may increase in some sporadic AD patients, as is the case of patients with familial AD, and (ii) that an elevated relative APL1β28 level in the CSF is a potential biomarker for sporadic AD. In the future, we plan to construct an assay system for measuring plasma APL1β and to perform large-scale/cohort studies to determine whether the relative APL1β level in CSF is useful for clinical diagnosis of AD. It will be interesting to determine when the ratio of APL1β28 production starts to rise in the pathological process of sporadic AD.

Using βAPP (Okochi et al, 2006; Weggen et al, 2001), mNotch-1 (Okochi et al, 2006) and APLP1, we found that fenofibrate and S2474 affect the γ-cleavage of multiple substrates. Whether GSMs bind to the TM of APLP1 remains to be determined. If this is the case, although some GSMs may target βAPP (Kukar et al, 2008), their binding would not be specific to βAPP.

Previously, we reported that Nβ, a Notch-1 Aβ-like peptide, is secreted (Okochi et al, 2006), and, in this study, we demonstrated that CSF contains as much APL1β as Aβ. This suggests that a novel group of physiological peptides (*i.e.* a group of Aβ-like peptides) may exist in the human brain. It seems possible that Aβ was identified first because high levels of Aβ accumulation are associated with AD. Because small brain peptides often have a variety of physiological roles, for example serving as neurotransmitters and ligands, it is important to further investigate the physiological functions of Aβ-like peptides such as APL1β and Nβ.

## MATERIALS AND METHODS

### Antibodies and reagents

Rabbit antisera OA601 and OA663 were raised against the synthetic peptides IQRDELAPAGTGVSRE and DELAPAGTGVSRE, respectively (MBL, Japan). The peptide sequences correspond to the sequence of the human APLP1 juxtamembrane domain (GenBank accession number, U48437). The monoclonal antibody 4G8 against Aβ was purchased from Signet, and the antiserum against APLP1 CTF was purchased from Calbiochem. HRP-conjugated anti-rabbit or anti-mouse IgG (Promega) was used as a secondary antibody. For detection of the biotinylated antibodies, we used neutravidin-HRP (Pierce). The Aβ40 peptide and the peptides DELAPAGTGVSREAVSGLLIMGAGG (APL1β25), DELAPAGTGVSREAVSGLLIMGAGGGS (APL1β27) and DELAPAGTGVSREAVSGLLIMGAGGGSL (APL1β28) were synthesized by Peptide Institute. Each peptide was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol, stored, and pretreated before use as described previously (Okochi et al, 2006). S2474 was synthesized by Shionogi. The secretase inhibitors L685458 and DAPT were purchased from Peptide Institute, and inhibitor IV and TAPI-1 were from Calbiochem.

### Collection of human CSF

Human CSF samples were collected in six facilities. All the experiments using CSF were approved by the ethical committee of Osaka University Hospital (Nos. 07139 and 07212). Lumbar punctures were performed using standard methods. Briefly, CSF (5–10 mL) was collected into sterile polypropylene tubes using atraumatic cannulas (21–23G) placed in the L3/L4 or L4/L5 intervertebral space. CSF was centrifuged for 10 min at 4,000 × *g*, and aliquots of the remaining CSF supernatants were frozen immediately at 80°C. The CSF samples were frozen and thawed once or twice before measurements.

### cDNA constructs

A cDNA encoding human APLP1 (Toyobo) was subcloned into pcDNA3.1 Hygro(+). The FAD mutant versions of PS1 were generated by PCR-based mutagenesis using the QuikChange-II kit (Stratagene) with wt PS1 cDNA as a template (Okochi et al, 2002).

### Cell culture and cell lines

SH-SY5Y neuroblastoma cells were grown in 1:1 DMEM/F12 media containing 10% fetal calf serum. HEK293 cells stably expressing either wt or mutant PS1, APLP1 and βAPP sw mutant were generated using Lipofectamine 2000 (Invitrogen) and cultured as described previously (Okochi et al, 2000b).

### Immunoblotting, combined IP-immunoblotting (IP-blot), and combined IP-autoradiography

For immunoblotting, cell lysates or brain FA-soluble fractions were prepared, diluted in SDS-sample buffer and separated by Tris-glycine or Tris-tricine (Invitrogen) SDS-PAGE as described previously (Okochi et al, 2000b). For analysis of proteins other than APL1β/Aβ, the proteins were transferred to a polyvinylidene difluoride membrane, and for analysis of APL1β/Aβ, the proteins were transferred to a nitrocellulose membrane and boiled for 10 min in phosphate buffered saline (PBS). Membranes were blocked in 5% skim milk and probed with 1:1,000 anti-APLP-1 C-terminal antibody, 1 μg/ml antibody 4G8 or 28 μg/ml biotinylated OA601 IgG, followed by probing with HRP-conjugated anti-rabbit IgG, anti-mouse IgG or neutravidin, respectively. Immunoreactive proteins were detected using ECL or ECL plus reagents (GE Healthcare), and chemiluminescence intensities were measured using a LAS3000 scanner, followed by analysis with Multi Gauge Ver3.0 software (Fuji Film). IP-blot analysis of cultured cell lysates expressing wt APLP1 or mutant PS1 was performed as described previously (Okochi et al, 1997). IP-autoradiography of

  

## The paper explained

### PROBLEM:

The 42 amino acid version of amyloid β-peptide (Aβ42) is the major constituent of senile plaques, which is the pathological hallmark of AD and is generated by proteolytic processing of the β-amyloid precursor protein (βAPP). Remarkably, in the CSF of AD patients Aβ42 levels are low, whereas this peptide accumulates within the brain. It is currently believed that this discrepancy may be due to the fact that Aβ42 is largely deposited in insoluble plaques within the brain and that clearance into the CSF is therefore reduced. Although the reverse relationship of Aβ42 levels can be used as a biomarker to some extent after disease onset, better surrogate markers specifically for presymptomatic diagnosis are desperately required.

### RESULTS:

In this study, we discovered APL1β28 as a novel and highly sensitive biomarker. This peptide is generated by the same proteolytic mechanism as Aβ42, except that it is derived from a divergent substrate, namely the βAPP-like protein, APLP1. Non-amyloidogenic APL1β28 can be detected in the CSF and its levels correlate with Aβ42 production. Remarkably, the ratio of APL1β28 to total APL1β are significantly increased in familial and sporadic AD cases.

### IMPACT:

We propose using the levels of APL1β28 as a surrogate marker for Aβ42 production in the central nervous system. This has clinical importance for the diagnosis and early detection of sporadic AD.

cultured media was performed as described previously (Okochi et al, 2002). Conditioned media collected from radiolabelled SH-SY5Y cells were adjusted to 50 mM Tris (pH 7.4), 1:500 protease inhibitor mix (Sigma) and 5 mM EDTA (pH 8.0) and then immunoprecipitated with OA601.

#### Combined IP/MALDI-TOF MS (IP/MS) analysis

Combined IP-MALDI-TOF MS analysis was carried out as described previously (Okochi et al, 2002). Either human CSF or conditioned media from SH-SY5Y cells was used. CSF (300 μl) was diluted with 700 μl of Tris buffer (pH 7.6) containing 5 mM EDTA and 1:500 protease inhibitor mix (Sigma). The MS peak heights and molecular masses were normalized to those of angiotensin and bovine insulin β-chain.

#### Radiolabelled pulse-chase experiment

Following treatments with 1 μM L685458, 10 μM DAPT, or 1 μM inhibitor IV for 16 h, SH-SY5Y cells were metabolically labelled for 8 h as described previously (Okochi et al, 2000a).

#### Preparation of FA-soluble fractions in human brains

Frozen brains were minced and suspended in two volumes of Tris buffered saline (TBS v/w) supplemented with protease inhibitor mix (Sigma). Suspensions were homogenized with a Teflon homogenizer and centrifuged at 100,000 × *g* for 15 min. The suspensions were extracted twice with 1% Triton X-100 and three times with 2% SDS in TBS, after which the 2% SDS-insoluble fractions were sonicated in FA and then centrifuged at 100,000 × *g* for 15 min. The resultant FA-soluble fractions were evaporated using a Speed-Vac, mixed with 2× SDS-sample buffer and heated for 5 min at 100°C.

#### Determination of amino acid sequences of APL1β peptides in human CSF

APL1β peptides immunoprecipitated from human CSF by OA601 were eluted with a 20% acetonitrile/0.1% FA solution. The eluates were passed through a 50-kDa filter unit (YM50 Microcon; Millipore), after which the filter was washed twice with the same solution. The resultant filtrates were applied to a C18 column (ODS-100V, TOSOH) using an auto-injection system (Agilent 1100 series). Subsequently, we performed LC/MS/MS analysis using a 50-min gradient (5 to 100% acetonitrile) at a flow rate of 200 μl/min (3200QTRAP; Applied Biosystems). The brain APL1β25, APL1β27 and APL1β28 species were eluted at ~20.7, 21.5 and 22.0 min, respectively. Enhanced mass scan, enhanced resolution and enhanced product ion analyses were performed after elution. All spectra for all peptides in CSF matched those of the synthetic APL1β25, APL1β27 and APL1β28 peptides. Turbo spray was used as an ion source and for each analysis, the curtain gas pressure, collision gas pressure, ion spray voltage, temperature, ion source gas 1 pressure and ion source gas 2 pressure were 15 pounds per squire inch (p.s.i.), 5 p.s.i., 5,500 V, 600°C, 50 and 50 p.s.i., respectively. The declustering potential, enhanced potential and collision cell exit potential were set at 70, 10 and 3 V, respectively. Analyst Version 1.4.1 software was used.

#### Quantitative LC/MS/MS analysis of APL1β species

Human CSF samples were pretreated using the ProteoSeek™ Albumin/IgG removal kit (PIERCE) according to the manufacturer's instructions. CSF, Cibacron Blue/Protein A gel slurry, and binding/wash buffer were mixed at 2:1:2 (v/v/v) ratio. The pretreated CSF samples were directly applied to a C18 column (ODS-100V; TOSOH) using an auto-injection system (Agilent 1100 series). Conditioned medium was

  

immunoprecipitated with OA601, and the precipitates were eluted with a solution of 20% acetonitrile and 0.1% FA. The eluates were passed through a 50-kDa filter unit (YM50 Microcon; Millipore). The resultant filtrates were applied to the C18 column. The HPLC parameters were as follows: mobile phase A, 0.1% FA; mobile phase B, acetonitrile with 0.1% FA; mobile phase gradient, 95% A–100% B in 50 min; flow rate, 200 μl/min. After LC/MS/MS analysis (3200QTRAP; Applied Biosystems), the peak areas of the daughter ions (b2, y20 and y21 for APL1β25/28; b2, y21 and y22 for APL1β27) were measured using the Multiquant™ software. The average of three calculated concentrations was defined as the concentration for each APL1β species in the CSF.

### Aβ and total tau ELISA

Aβ40 or Aβ42 levels in human CSF or conditioned media were quantified using commercial sandwich ELISA kits (WAKO Pure Chemical Industries, Ltd.). Total tau levels in human CSF were quantified using commercial sandwich ELISA kits (Innogenetics, Belgium). After the addition of the protease inhibitor mixture, human CSF and conditioned media (Sigma) were appropriately diluted with the standard diluents provided in the kits.

### *In vitro* BACE cleavage assay

Recombinant ectodomain BACE1/2 was obtained from R&D Systems, and the synthetic fluorescent peptides wt APP (Nma-EVKM-DAEFK(Dnp)-RR-$NH_2$) and APLP1 (Nma-EIQR-DELAK(Dnp)-RR-$NH_2$) were obtained from Peptide Institute Co. Ltd. Recombinant BACE1 or BACE2 (7.4 nM) was mixed with the substrate (1 μM) in the reaction buffer (50 mM sodium acetate, pH 5.0, 0.008% Triton X-100) in a 96-well microtiter plate. The level of fluorescence in each well was determined using a fluorometer (Spectra Max GeminiXS), and the data were analysed using SOFTmax PRO (Molecular Devices).

### Fibril formation by APL1β

Fibril formation of Aβ peptide and SEC were previously described (Hartley et al, 1999). Briefly, Aβ1-40, APL1β25, APL1β27 and APL1β28 peptides were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol to a concentration of 1 mg/ml. Before use, the peptides were dried using a Speed-Vac and dissolved in 1 mM NaOH to a concentration of 1 mM. Solutions were immediately adjusted to pH 7.0 with 10 mM NaOH and diluted to a final concentration of 500 μM in 1:1 water/PBS. Peptides were then incubated at 37°C for 0–48 h and 5-μl aliquots were collected every 4 h. Peptides were diluted with 15 μl of PBS and then centrifuged for 10 min at 16,000 × *g*. Supernatants were fractionated by SEC, and precipitated fibrils were analysed by a transmission electron microscopy (TEM).

### SEC

Ten μl of diluted supernatants (protofibrils and low molecular weight peptides) were separated by a Superdex 75 size exclusion column (Hartley et al, 1999) using an AKTA explorer 10S system (GE Healthcare). Peptides were eluted with 50 mM Tris pH 7.6 containing 150 mM NaCl. Elution was monitored by measuring the absorbance at 215 and 280 nm.

### TEM

Precipitated fibrils were suspended in 10 μl of PBS. Samples (3 μl) were applied to carbon-coated Maxtaform HF36400 mesh grids (Ohkenshoji) and incubated for 3 min. Excess samples were absorbed with filter paper, after which an equal volume of uranyl acetate solution was added. After incubation for 2 min, the solution was removed and the grid was air-dried. Samples were examined using a Hitachi H-7650 transmission electron microscope.

### Immunohistochemistry

Formalin-fixed, paraffin-embedded brain blocks were obtained for immunohistochemistry. Sections (10 μm) cut from these were deparaffinized and pretreated with 0.5% $H_2O_2$ in PBS for 30 min. The primary antibody was diluted in PBS containing 0.1% Triton X-100 (PBST) and 1% normal serum from the species in which the secondary antibody was raised. After incubation with a specific primary antibody for 48 h at 4°C, the sections were treated with the appropriate biotinylated secondary antibody at 1:1,000 concentration (Vector Laboratories) for 2 h at room temperature. The sections were then washed in PBST and incubated for 2 h at room temperature with avidin-biotinylated HRP complex (ABC Elite; Vector Laboratories). Peroxidase labelling was detected by incubation with a solution containing 0.01% 3,3′-diaminobenzidine (Sigma), 0.6% nickel ammonium sulfate, 0.05 M imidazole and 0.00015% $H_2O_2$. Negative control experiments were performed as described previously but in the absence of a primary antibody.

## Author contributions

KY and MO detected APL1β species in CSF; TSK determined amino acid sequence of APL1β species by LC/MS/MS; ST, TN and TSK measured APL1β by LC/MS/MS; KN, MO and KY performed pulse-chase experiments; MO, JJ and KY performed *in vitro* fibril/protofibril formation assay; KY and ST performed *in vitro* BACE cleavage assay; KY, KN and KM performed Aβ ELISA assay; ST and KY generated PS1 FAD mutant expressing cells; TA performed immunohistochemical study; TI, KK, TT, MK, MI, KD, HK, TT, TM, RH, KT, HA, RK, KT, MT, ST and MO collected CSF samples; HS and CH provided analytical tools and reagents; MO conceived and designed the study, and analysed the data; MO wrote the paper with contributions from HS, CH and ST.

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