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Altered γ-secretase activity in mild cognitive impairment and Alzheimer's disease

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We investigated why the cerebrospinal fluid (CSF) concentrations of Aβ42 are lower in mild cognitive impairment (MCI) and Alzheimer's disease (AD) patients. Because Aβ38/42 and Aβ40/43 are distinct product/precursor pairs, these four species in the CSF together should faithfully reflect the status of brain γ-secretase activity, and were quantified by specific enzyme-linked immunosorbent assays in the CSF from controls and MCI/AD patients. Decreases in the levels of the precursors, Aβ42 and 43, in MCI/AD CSF tended to accompany increases in the levels of the products, Aβ38 and 40, respectively. The ratios Aβ40/43 *versus* Aβ38/42 in CSF (each representing cleavage efficiency of Aβ43 or Aβ42) were largely proportional to each other but generally higher in MCI/AD patients compared to control subjects. These data suggest that γ-secretase activity in MCI/AD patients is enhanced at the conversion of Aβ43 and 42 to Aβ40 and 38, respectively. Consequently, we measured the *in vitro* activity of raft-associated γ-secretase isolated from control as well as MCI/AD brains and found the same, significant alterations in the γ-secretase activity in MCI/AD brains.

INTRODUCTION

Senile plaques, the neuropathological hallmark of Alzheimer's disease (AD), are composed of amyloid β-protein (Aβ). Aβ is derived from β-amyloid precursor protein (APP) through sequential cleavage by β- and γ-secretases. β-Secretase cleaves at the luminal portion (β-site) of APP to generate a β-carboxyl terminal fragment of APP (βCTF), an immediate substrate of γ-secretase, to produce different Aβ species (for a review see Selkoe, 2001). The most abundant secreted Aβ species is Aβ40,

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whereas the species that has two extra residues (Aβ42) is a minor one (<10%); however, the latter is the one that deposits first and predominates in senile plaques (Iwatsubo et al, 1994).

Presenilin 1/2 make up the catalytic site of γ-secretase. The enzymatic properties of γ-secretase that cleave the transmembrane domain of βCTF have been an enigma, although recent studies provided partial elucidation of this mechanism (Qi-Takahara et al, 2005; Takami et al, 2009). γ-Secretase has two product lines, which successively convert the Aβ49 and Aβ48 that are generated by ε-cleavage, to shorter Aβs by releasing tri- or tetrapeptides in a stepwise fashion. Aβ49 is successively cleaved mostly into Aβ40 via Aβ46 and Aβ43, while Aβ48 is similarly cleaved into Aβ38 via Aβ45 and Aβ42 (see Fig 1). Importantly, the differences between the amounts of released tri- and tetrapeptides determine the levels of the different Aβ species produced (Takami et al, 2009). Thus, the true activity of γ-secretase is defined by the amounts of tri- and tetrapeptides released, but not by the amounts of Aβ species produced. Of note, the most abundant species Aβ40 is derived not from Aβ42, but from Aβ43. Also Aβ38 is derived mainly from Aβ42 (Fig 1). The longer Aβs in cerebrospinal fluid (CSF) including Aβ49 and 46 as well as Aβ48 and 45 must be generated at negligible levels, but may neither be secreted to the interstitial fluid (ISF) nor recruited to CSF. This suggests that the status of brain, and possibly neuronal, γ-secretase could be accurately assessed by measuring all four Aβ species generated by the two product lines of γ-secretase.

Using enzyme-linked immunosorbent assays (ELISAs), we quantified Aβ40 and 43 and Aβ38 and 42 in CSF samples from control subjects and mild cognitive impairment (MCI)/AD patients. The CSF concentrations of Aβ43 and Aβ42 were found to be significantly lower in MCI/AD compared with controls. The ratio of Aβ38/42, which represents the ratio of product/precursor and thus the cleavage efficiency of Aβ42, was plotted against the ratio of Aβ40/43, which represents the ratio of product/precursor in the other product line and thus the cleavage efficiency of Aβ43. The ratio of Aβ38/42 was largely proportional to that of Aβ40/43, indicating that the two cleavage processes are tightly coupled, but both were generally higher in MCI/AD patients compared to control subjects These results

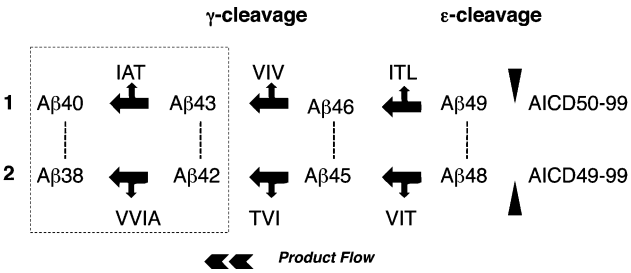

Figure 1. Generation of Aβs through stepwise processing of βCTF. At the first step, βCTF is cleaved at the membrane-cytoplasmic boundary (ε-cleavage), producing AICD (APP intracellular domain) 50–99 and 49–99. Counterparts Aβ49 and 48 in turn are cleaved in a stepwise fashion, releasing tri- and tetrapeptides. One product line converts Aβ49 mostly to Aβ40 via Aβ46 and Aβ43. The other product line converts Aβ48 to Aβ38 via Aβ45 and Aβ42. It should be noted that the differences between the amounts of released tri- or tetrapeptide determine the amounts of Aβs produced. Broken lines indicate corresponding Aβs on the two product lines.

suggest that the activity of brain γ-secretase in MCI/AD is enhanced at the conversion of Aβ43 to Aβ40 and Aβ42 to Aβ38, which would result in significantly lower CSF concentrations of Aβ42 and 43. In support of this hypothesis, the activities of raft-associated γ-secretase from control and MCI/AD brains were found to be significantly different: although the total Aβ production was similar, the γ-secretase in MCI/AD brains produced significantly larger ratios of Aβ40/43 and Aβ38/42 than the enzyme in control brains. This raises the possibility that lower CSF levels of Aβ42 and 43 simply reflect the altered γ-secretase activity in the MCI/AD-affected brains.

RESULTS

The CSF concentrations of Aβs were in the following order: Aβ40 > Aβ38 > Aβ42 ≫ Aβ43 in all CSF samples examined (Table 1 and Supporting Information Fig S2A). The relative amounts of Aβs were constant across the samples: Aβ38:40 ratio in CSF was ~1:3, and Aβ42:43 ratio was ~10:1. The CSF

Table 1. Subject characteristics and CSF concentrations of Aβs

	Control	MCI	AD	ANOVA ***p-value
Age (years)	74.9 ± 7.5	72.5 ± 6.6	72.3 ± 8.2	
N (male/female)	21 (10/11)	19 (7/12)	24 (7/17)	
MMSE score	28.7 ± 1.9	25.7 ± 2.6	19.6 ± 3.3	
ApoE ε4	3 (14.3%)	10 (52.6%) [a]	14 (58.6%) [a]	
Aβ38 (pM)	594.5 ± 286.3	669.4 ± 247.6	760.57 ± 269.4	
Ln(Aβ38)	6.28 ± 0.46	6.44 ± 0.38	6.56 ± 0.41	NS
Aβ40 (pM)	1607.9 ± 712.9	1939.5 ± 698.0	2292.6 ± 799.6	
Ln(Aβ40)	7.28 ± 0.47	7.51 ± 0.38	7.68 ± 0.35	0.007
Aβ42 (pM)	133.1 ± 53.4	83.2 ± 49.4**	90.3 ± 40.1 [a]	
Ln(Aβ42)	4.80 ± 0.47	4.25 ± 0.60	4.40 ± 0.47	0.004
Aβ43 (pM)	11.8 ± 5.7	6.8 ± 5.6**	7.0 ± 4.6**	
Ln(Aβ43)	2.32 ± 0.60	1.59 ± 0.86	1.76 ± 0.62	0.004

[a]2 MCI subjects were homozygous for ε4, while 4 AD subjects were homozygous for the allele.
**$p < 0.05$; Dunnett's *t*-test after log-transformation for comparing between control and MCI or AD.
***p-value of analysis of variance after log-transformation.

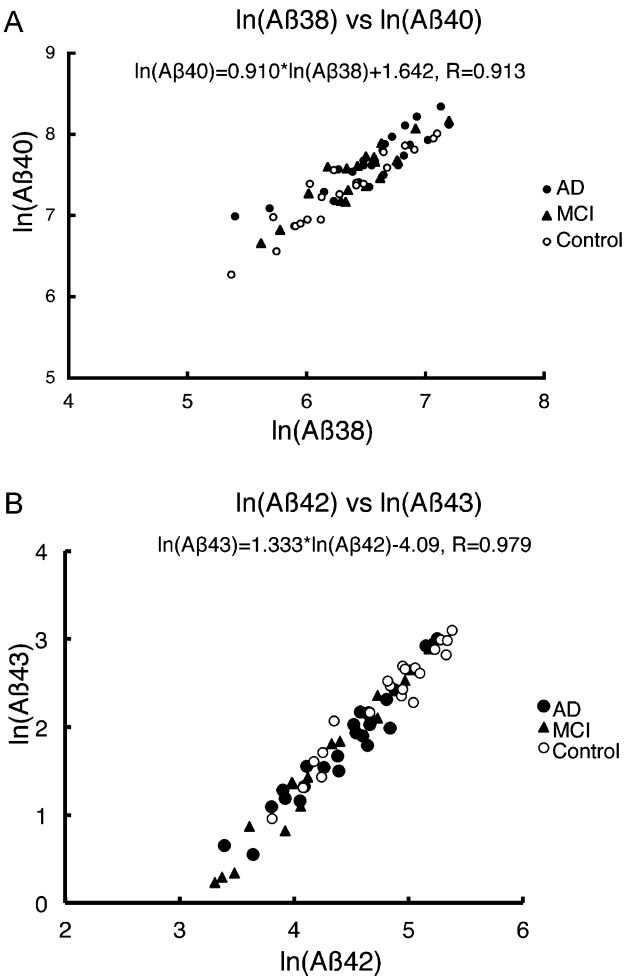

Figure 2. Relationships between the levels of Aβ40 and 38, and between those of Aβ43 and 42 in CSF from controls and MCI/AD patients.

A. The levels of ln(Aβ40) were proportional to those of ln(Aβ38) ($\ln(A\beta40) = 0.910 \times \ln(A\beta38) + 1.642$, $R = 0.913$).

B. The levels of ln(Aβ43) were proportional to those of ln(Aβ42) ($\ln(A\beta43) = 1.333 \times \ln(A\beta42) - 4.09$, $R = 0.979$). It should be noted that the levels of both ln(Aβ42) and ln(Aβ43) in MCI [filled triangle ($n = 19$)]/AD [filled circle ($n = 24$)] are lower than those in controls [open circles ($n = 21$)].

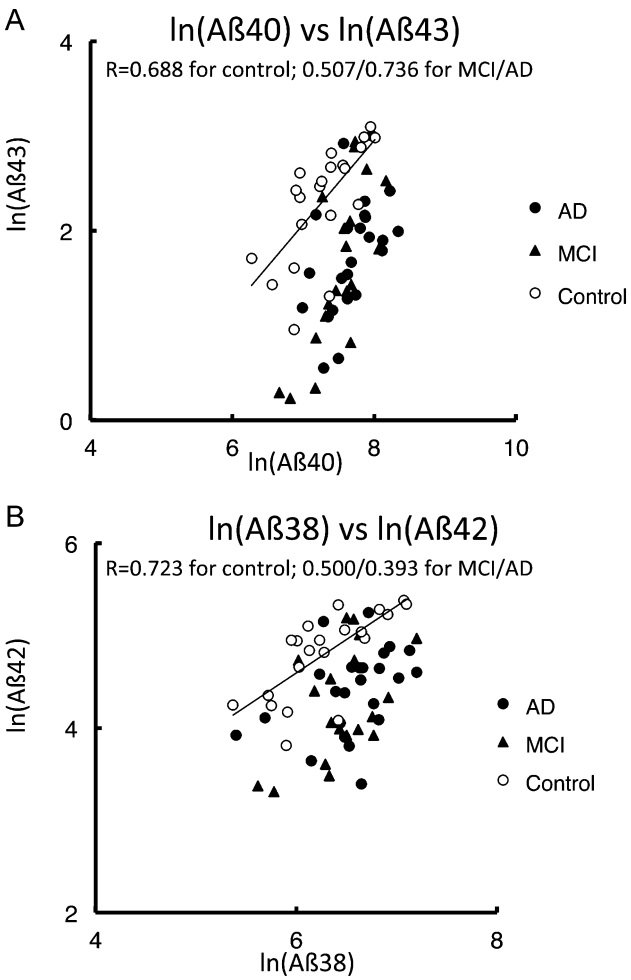

Figure 3. Relationships between the levels of Aβ43 and 40, and between those of Aβ42 and 38 in CSF from controls (open circles) and MCI (closed triangle)/AD patients (closed circle).

A. The levels of ln(Aβ43) correlate with those of ln(Aβ40) within controls ($R = 0.688$), and barely within MCI/AD subjects ($R = 0.507/0.736$). The plots for MCI/AD were located below the regression line for control ($p < 0.001$, ANOVA).

B. The levels of Aβ42 correlate with those of Aβ38 within controls ($R = 0.723$), and barely within MCI/AD ($R = 0.500/0.393$). The plots for MCI/AD were situated below the regression line for controls ($p < 0.001$, ANOVA).

concentrations of Aβ40 were significantly increased in AD compared to control (Table 1; $p < 0.05$, Dunnett's *t*-test). Additionally, the CSF concentrations of Aβ38 tended to be increased in AD patients compared to controls. In contrast, those of Aβ42 and 43 were significantly decreased in MCI/AD compared to controls ($p < 0.05$, Dunnett's *t*-test). Interestingly, as reported previously (Schoonenboom et al, 2005), the CSF concentrations of Aβ40 and Aβ38 were proportional to each other in all subjects [Fig 2A; $\ln(A\beta40) = 0.910 \times \ln(A\beta38) + 1.642$, $R = 0.913$, where ln(Aβ40) is the logarithm of Aβ40], even in MCI/AD cases. This was despite the fact that these species are derived from and the final products of the two different product lines of γ-secretase activity (Fig 1; Takami et al, 2009). In other words, the amounts of products in the third step of cleavage were strictly proportional to each other across the product lines.

Aβ42 and Aβ43 are produced by the second cleavage step of each product line. Like Aβ40 and Aβ38, the CSF concentrations of Aβ42 and Aβ43 are also proportional to each other in controls and in MCI/AD patients [Fig 2B; $\ln(A\beta43) = 1.333 \times \ln(A\beta42) - 4.09$, $R = 0.979$]. On the other hand, the levels of Aβ43 and Aβ40 (a precursor and its product) were correlated in control [Fig 3A; $\ln(A\beta43) = 0.884 \times \ln(A\beta40) - 4.118$, $R = 0.688$] and in MCI/AD subjects ($R = 0.507/0.736$ for MCI/AD, respectively) but the MCI/AD values were located below the regression line for controls and thus provided lower Aβ43 measures compared with controls for a given Aβ40 measure (Fig 3A; $p < 0.001$, analysis of variance, ANOVA). Conversely,

for a given Aβ43 value, the plot provided a higher Aβ40 measure in MCI/AD cases. There was a similar situation for the levels of Aβ42 and Aβ38. The levels of Aβ42 and Aβ38 were correlated each other in control subjects [Fig 3B; $\ln(\text{Aβ42}) = 0.724 \times \ln(\text{Aβ38}) + 0.251$, $R = 0.723$], but barely in MCI/AD ($R = 0.500$ for MCI; 0.393 for AD), and the MCI/AD plots were situated below the regression line for controls ($p < 0.001$, ANOVA). For a given Aβ42 value, the plot provided a higher Aβ38 measure in MCI/AD compared with controls.

These lower concentrations of Aβ42 appeared to be compensated with higher concentrations of Aβ38 as the levels of $\ln(\text{Aβ38} + \text{Aβ42})$ did not vary even in MCI/AD ($p = 0.293$, ANOVA). Thus, this points to the possibility that more Aβ42 and Aβ43 are converted to Aβ38 and Aβ40, respectively, in MCI/AD brains. According to numerical simulation based on the stepwise processing model, as the levels of βCTF decline to null, the levels of Aβ43 and 42 decrease and the ratios of Aβ40/43 and Aβ38/42 increase (unpublished observation). However, this situation can be excluded as the mechanism for lower concentrations of Aβ42 and 43, because the levels of βCTF have never been reported to be reduced in AD brains nor in plaque-forming Tg2576 mice that show lower CSF Aβ42 concentrations (Kawarabayashi et al, 2001). Thus, it is reasonable to suspect that the final cleavage steps from Aβ43 mostly to 40 and from Aβ42 to 38 are significantly enhanced in parallel (increases in released tri- and tetrapeptides) in brains affected by MCI/AD compared with controls (Fig 1).

This relationship in γ-secretase cleavage becomes clearer by plotting the product/precursor ratio representing cleavage efficiency at the step from Aβ42 to 38 (Aβ38/42) against that representing the cleavage efficiency at the step from Aβ43 to 40 (Aβ40/43) (Fig 4). The 'apparent' cleavage efficiency of Aβ43 was approximately 40-fold larger than that of Aβ42. The two ratios in CSF samples from MCI/AD and control subjects were largely proportional to each other, indicating that the corresponding cleavage processes in the two lines are tightly coupled (Fig 4). All plots were situated on a distinct line [$\ln(\text{Aβ38/42}) = 0.748 \times \ln(\text{Aβ40/43}) - 2.244$, $R = 0.936$] and its close surroundings. An increase in the cleavage from Aβ43 to 40 (*i.e.* more Aβ43 is converted to Aβ40) accompanied an increase in the cleavage from Aβ42 to 38 and *vice versa*, although the mechanism underlying this coupling between the two product lines remains unknown. This reminds us of the 'NSAID effect' in the 3-([3-cholamidopropyl]dimetylammonio)-2-hydroxy-1-propanesulfonate (CHAPSO)-reconstituted γ-secretase system (Takami et al, 2009; Weggen et al, 2001) in which the addition of sulindac sulfide to the γ-secretase reaction mixture, as expected, significantly suppressed Aβ42 production and increased Aβ38 production presumably by increasing the amounts of released tetrapeptide (VVIA) (Takami et al, 2009) and other peptides.

Most importantly, this graph provides a clear distinction between the control and MCI/AD groups (Fig 4; Aβ40/43 for MCI/AD *vs*. control, $p = 0.000$; Aβ38/42 for MCI/AD *vs*. control, $p = 0.000$; ANOVA, followed by Dunnett's *t*-test). The control values plotted close to the origin, whereas those for MCI/AD patients were distant from the origin along the line [$\ln(\text{Aβ38/42}) = 0.748 \times \ln(\text{Aβ40/43}) - 2.244$, $R = 0.936$]. It is also of note

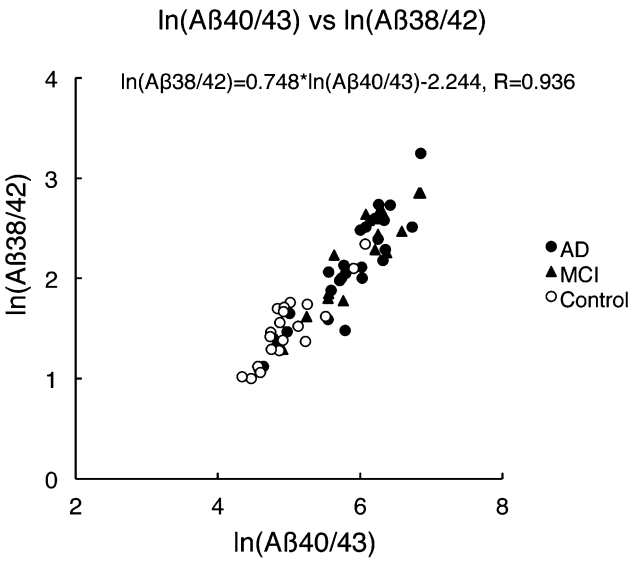

Figure 4. Ln(Aβ40/43) *versus* ln(Aβ38/42) plot. The ratios represent the cleavage efficiency at the final step of each line. Both ratios are largely proportional to each other ($y = 0.748 \times -2.244$, $R = 0.936$) and plots are located on the line and its close surroundings. This plot clearly distinguishes between control subjects and MCI/AD patients (Aβ40/43 for MCI *vs*. control, $p = 0.000$; Aβ38/42 for MCI *vs*. control, $p = 0.000$; ANOVA, followed by Dunnett's *t*-test). Control plots [open circles ($n = 21$)] are located close to the origin and MCI/AD plots [closed triangles ($n = 19$) and closed circles ($n = 24$), respectively] are a little distant from the origin.

that there was no significant difference between MCI and AD patients (Fig 4; Aβ40/43 for AD *vs*. MCI, $p = 1.000$; Aβ38/42 for AD *vs*. MCI, $p = 1.000$; Bonferroni's *t*-test). Two control values were a little farther from the origin, which may suggest that these subjects already have latent Aβ deposition or are in the preclinical AD stage. Additionally, we examined quite a small number of CSF samples from presenilin (PS) 1-mutated (symptomatic) familial AD (FAD) patients (T116N, L173F, G209R, L286V and L381V). Out of the three FAD cases near the regression line, two (T116N and L286V) were distant from the origin like sporadic AD cases and one (L381V) was closer to the origin than controls (both Aβ42/43 levels were lower than control; unpublished data). The remaining two (G209R and L173F) were extremely displaced from the line. Thus, a larger number of FAD cases are needed to give an appropriate explanation for their unusual characteristics in the plot, and the alteration of CSF Aβs shown above seems to be applicable only for sporadic AD.

Altogether, in MCI/AD, more Aβ42 and 43 are processed to Aβ38 and 40, respectively, than in controls. Even in MCI/AD, strict relationships are maintained between the levels of Aβ42 and Aβ43, and between those of Aβ38 and Aβ40 as seen in controls, which are predicted by the stepwise processing kinetics (unpublished observation). Thus, our observations suggest that lower CSF concentrations of Aβ42 and 43 and presumably higher CSF concentrations of Aβ38 and 40 are the consequence of altered γ-secretase activity in brain rather than the effect of preferential deposition of the two longer Aβ species (Aβ42 and 43) in senile plaques, which would not have maintained such strict relationships between the four Aβ species in CSF.

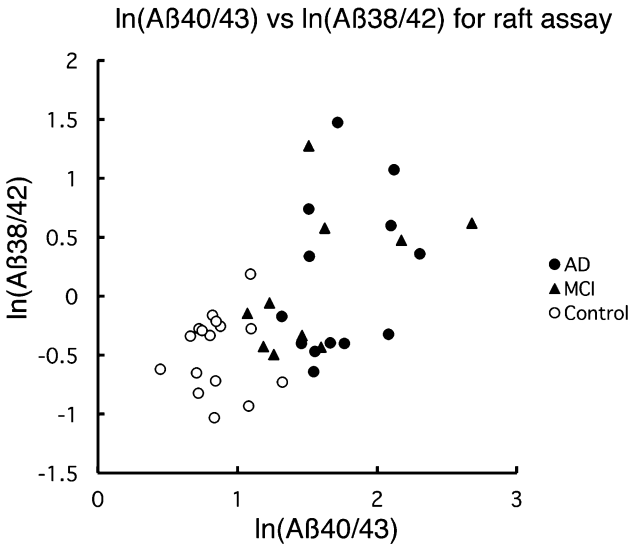

Figure 5. Ln(Aβ40/43) *versus* ln(Aβ38/42) plot based on direct quantification of raft-associated γ-secretase activity. The raft-associated γ-secretase prepared from control and MCI/AD brain specimens was incubated with βCTF for 2 h at 37°C (see Materials and Methods Section). Produced Aβs were quantified by Western blotting using specific antibodies. This plot distinguishes between control subjects and MCI/AD patients (Aβ40/43 for control *vs.* MCI/AD, $p < 0.001$; Aβ38/42 for control *vs.* MCI/AD, $p = 0.001$; Welch's *t*-test). MCI/AD plots [closed triangles ($n = 10$) and closed circles ($n = 13$), respectively] are as a whole a little distant from the origin, whereas control plots [open circles ($n = 16$)] are close to the origin.

To further test our hypothesis, we directly measured γ-secretase activities associated with lipid rafts isolated from AD, MCI and control cortices (Brodmann areas 9–11). For definite confirmation of the Aβ species, the reaction mixtures were subjected to quantitative Western blotting using specific antibodies rather than ELISA. At time 0, deposited Aβ42/43 species were detected in rafts from MCI/AD brains but not in control specimen (Supporting Information Fig S3). The amounts of ln(Aβ38 + Aβ42), which reflect the total capacity of the Aβ38/42-producing line, did not vary between AD, MCI and controls (Supporting Information Fig S4; $p = 0.969$, ANOVA). Thus, the gross activities of raft γ-secretase were comparable among the three groups. However, the plotted values for Aβ40/43 *versus* Aβ38/42 are divided into two groups: MCI/AD and controls (Fig 5; Aβ40/43 for control *vs.* MCI/AD, $p < 0.001$; Aβ38/42 for control *vs.* MCI/AD, $p = 0.001$; Welch's *t*-test) in the same way as those derived from CSF (Fig 4). It is notable that Figs. 4 and 5 are based on different methods, ELISA and Western blotting, respectively, but give similar results. There were no significant differences between MCI and AD specimen, although MCI patients (91 ± 4.9-year-old) were older than controls (77 ± 6.5-year-old) or AD patients (80 ± 5.0-year-old) (Aβ40/43 for MCI *vs.* AD, $p = 0.342$; Aβ38/42 for MCI *vs.* AD, $p = 0.911$). There were similar significant differences between control *versus* AD in the groups of which the ages were not significantly different (Aβ40/43 for control *vs.* AD, $p < 0.001$; Aβ38/42 for control *vs.* AD, $p = 0.03$).

DISCUSSION

Here, we assume that (i) Aβs in CSF are produced exclusively by γ-secretase in the brain, possibly in neurons; and (ii) Aβs in CSF are in the steady state. With these assumptions, the combined measurement of four Aβ species in CSF should predict the activity of γ-secretase in the brain. Here, the alterations in the γ-secretase activities do not mean the gross activity, *i.e.* total Aβ production, but the cleavage efficiency of the intermediates, Aβ42 and Aβ43.

In the present study, we quantified in CSF the four Aβ species, Aβ38/42 and Aβ40/43, but the Western blotting indicated the presence of additional Aβ species, Aβ37 and 39, in CSF (Supporting Information Fig S2). At present, we cannot exclude the possibility that a certain carboxyl terminus-specific protease(s) in CSF acts on the pre-existing Aβ species and converts them to Aβ37 and 39 (Zou et al, 2007). However, according to our unpublished data (Takami et al, unpublished observations), it is plausible that Aβ37 is derived from Aβ40, whereas Aβ39 is derived from Aβ42. Even if so, these pathways are very minor (~20–100-fold less) compared to the two major pathways, Aβ42 to Aβ38, and Aβ43 to Aβ40, when assessed by a reconstituted system (Takami et al, 2009). Thus, such strict relationships between four Aβs may have been relatively independent of Aβ37 and 39. The detailed relationship between all Aβs in the CSF awaits further quantification of the additional two Aβ species.

Currently, we do not know why the observation that Aβ40 is higher in MCI/AD CSF has so far not been reported except a recent paper (Simonsen et al, 2007). In fact, some of us previously reported no significant differences in CSF Aβ40 between AD and control subjects using a different ELISA (Shoji et al, 1998). It may be notable that we used newly constructed ELISA for Aβ40 based on a different set of monoclonal antibodies and thus, those discrepancies may come from the different antibody/epitope combination used for ELISA and/or different assay methods. In particular, it should be noted that all ELISAs used here detect Aβ1-x only, but not amino-terminally truncated forms. In this context, the ratio of Aβ40/43 appears to be more informative to discriminate between control and MCI/AD than the absolute levels of Aβ40 alone (Table 1 and Fig 5). It is possible that even if Aβ40 is not different between control and MCI/AD, the ratio Aβ40/43 could discriminate them.

We are the first to measure CSF Aβ43 using ELISA. The CSF concentrations of Aβ43 are 10-fold less than those of Aβ42. Nevertheless, the specificity of the newly constructed ELISA made the quantification of accurate levels of Aβ43 possible (Supporting Information Fig S1). Regarding the Aβ43 measures, we observed that its behaviour is entirely similar to that of Aβ42 in MCI/AD. Our preliminary observations using immunocytochemistry and ELISA quantification strongly suggest that Aβ43 deposits in aged human brains at the same time as Aβ42 (unpublished observations). Furthermore, Saido and colleagues have only recently reported that a PS1 R278I mutation in mice (heterozygous) caused an elevation of Aβ43 and its early and pronounced accumulation in the brain (Saito et al, 2011). It is possible that the cleavage of βCTF by this R278I γ-secretase may

be profoundly suppressed in the third cleavage step of the product line 1 (see Fig 1), which would result in negligible levels of Aβ40 and unusually high levels of Aβ43 (Nakaya et al, 2005). These results suggest that the role of Aβ43 should be reconsidered for the initiation of β-amyloid deposition and thus in AD pathogenesis.

Lower CSF concentrations of Aβ42 and 43 are not exclusively limited to MCI/AD. For example, similar low concentrations of Aβ42 and 43 were found in the CSF from eight patients with idiopathic normal pressure hydrocephalus (iNPH) (Aβ42, 76.3 ± 37.3 pM, $p = 0.012$ compared to controls: Aβ43, 5.2 ± 2.9 pM, $n = 8$, $p = 0.004$ compared to controls: Bonferroni's *t*-test; Silverberg et al, 2003). Thus, lower CSF concentrations of Aβ42 and 43 alone were unable to distinguish between iNPH and MCI/AD, and further, it is claimed that the former is often associated with abundant senile plaques, raising the possibility that Aβ deposition is enhanced by iNPH (Silverberg et al, 2003). However, when their partners Aβ38 and 40 were measured in CSF, both were found not to be significantly increased in iNPH (Aβ38, 459.2 ± 138.5 pM, $p = 0.484$ compared to controls; Aβ40, 1094.4 ± 375.3 pM, $n = 8$, $p = 0.103$ compared to controls; Table 1) in sharp contrast to MCI/AD indicating that the cleavage in iNPH at the steps from Aβ43 to 40 and from Aβ42 to 38 is not enhanced as it is in MCI/AD. Thus, it may be that the dilution effect elicited by ventricular enlargement would be the cause of lower CSF Aβ42 and 43 found in iNPH.

Currently, we do not know the mechanism behind the altered activity of brain γ-secretase in MCI/AD (Fig 4). First, it is of note that rafts prepared from MCI/AD brains but never from control brains at SP stage 0/A accumulated Aβ42 and Aβ43 (Supporting Information Fig S3; Oshima et al, 2001). It is possible that raft-deposited Aβ42/43 could induce a change in the γ-secretase activity, although the extent of the alteration in the activity appears not to be related to the extent of accumulation (unpublished observation). In this regard, it is of interest to note that Tg2576 mice, the best characterized AD animal model, shows reduced levels of Aβ42 in plasma as well as in CSF at the initial stage of Aβ deposition (Kawarabayashi et al, 2001). If the assumption here is correct, this may suggest that γ-secretase that produces plasma Aβs could also be altered. However, thus far, we have failed to replicate significantly lower Aβ42 levels or Aβ42/Aβ40 ratios in plasma from AD patients.

Second, there could be heterogenous populations of γ-secretase complexes that have distinct activities due to subtle differences in their components. γ-Secretase is a complex of four membrane proteins including PS, nicastrin (NCT), anterior pharynx defective 1 (Aph1) and presenilin enhancer 2 (Pen 2) (Takasugi et al, 2003). Aph 1 has three isoforms, and each can assemble active γ-secretase together with other components (Serneels et al, 2009). NCT, a glycoprotein, is present in immature and mature forms (Yang et al, 2002). The abundance of these heterogenous populations of proteins in the brain is probably under strict control. During MCI/AD, a certain population could replace other populations of γ-secretase and thus may show a distinct activity as a whole.

The data shown here represent only a cross-sectional study, but our keen interest is how the CSF levels of the four Aβ species would shift during the longitudinal course in an individual who is going to develop sporadic AD. Does one have any period during life when Aβ42 and 43 are at higher levels in CSF, and thus the ratios of Aβ38/42 and Aβ40/43 are smaller? At this period when the final cleavage steps of γ-secretase would be suppressed, the ISF concentrations of Aβ43 and 42 would increase, which would start or promote their aggregation in the brain parenchyma. If so, during life span, the individual's plot would move down along the regression line and move up as senile plaques accumulate, and the individual would eventually develop sporadic AD. However, thus far the period when there are increases in CSF Aβ42/43 has never been reported for sporadic AD. Nor has it been reported for asymptomatic FAD carriers (Ringman et al, 2008), whereas their plasma is known to contain higher levels (and percent) of Aβ42 (Kosaka et al, 1997; Ringman et al, 2008; Scheuner et al, 1996). It is likely that the stage of normal cognition and Aβ accumulation already accompanies reduced CSF Aβ42. If so, the alterations of γ-secretase should continue on for decades. Most interestingly, this alteration of CSF Aβ regulation seems to be planned to prevent further accumulation of Aβ42 and 43 in the brain.

However, Hong et al (2011) have recently shown, using *in vivo* microdialysis to measure ISF Aβ in APP transgenic mice, that the increasing parenchymal Aβ is closely correlated with decreasing ISF Aβ, suggesting that produced Aβ42 is preferentially incorporated into existing plaque-Aβ. This is a prevailing way of the interpretation of the data. Another way of the interpretation of data would be that during aging from 3 to 24 months, γ-secretase activity becomes altered and produces decreasing amounts of Aβ but with an increasing ratio of Aβ38/42 (and Aβ40/43). It is worth to mention that produced Aβ42 (but not Aβ40) appears to be selectively bound to rafts (from CHO cells) after long incubation (>4 h; Wada et al, unpublished observation). Also of note is that we quantified the total (free and bound) Aβ produced by an *in vitro* reconstituted system (Fig 5). What is claimed here is that decreased levels of CSF Aβ42 are largely due to alterations of γ-secretase activity rather than due to selective deposition of Aβ42 in preexisting plaques. What proportions of decreased ISF (CSF) Aβ42 levels would be contributed to by altered γ-secretase activity and selective deposition of Aβ42/43 to parenchymal plaques awaits future studies.

Finally, our observation has therapeutic implication. As shown elsewhere and here above, if Aβ42 is the culprit for MCI/AD, non-steroidal anti-inflammatory drugs (NSAIDs) would have been quite a reasonable therapeutic compound, which enhances cleavage at the third step in the stepwise processing, leading to lower levels of Aβ42 without greatly interfering with the Aβ bulk flow (Weggen et al, 2001). This sharply contrasts with some of the γ-secretase inhibitors currently under development and in clinical trial, which block the Aβ bulk flow. However, the present study raises the possibility that even if NSAIDs are administered, the expected beneficial effect could be minimal in MCI/AD patients, because in these patient brains, γ-secretase is already shifted to an NSAID-like effect.

MATERIALS AND METHODS

Subjects

Cerebrospinal fluid samples from 24 AD patients (mild to moderate AD; 50–86 years old), 19 MCI patients (57–82 years old) and 21 control subjects (61–89 years old) were collected (see Table 1) at Department of Neurology, Hirosaki University Hospital and at Department of Geriatrics and Gerontology, Tohoku University Hospital, and at Department of Neurology, Niigata University Hospital. The CSF samples from (symptomatic) 5 FAD (mPS1) patients (T116N, L173F, G209R, L286V and L381V) were from Niigata University Hospital. Probable AD cases met the criteria of the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders (NINCDS-ADRDA) (Kuwano et al, 2006; McKhann et al, 1984). Additional diagnostic procedures included magnetic resonance imaging. Dementia severity was evaluated by the Mini-Mental State Examination (MMSE). Diagnosis of MCI was made according to the published criteria (Winblad et al, 2004). Diagnosis of iNPH was made according to the guideline issued by the Japanese Society of NPH (Ishikawa et al, 2008). Controls who had no sign of dementia and lived in an unassisted manner in the local community were recruited. All individuals included in this study were Japanese and 24 AD patients examined here were judged to have sporadic AD because of negative family history. This study was approved by the ethics committee at each hospital or institute.

Human cortical specimens for quantification of raft-associated γ-secretase activity were obtained from those brains that were removed, processed and placed in −80°C within 12 h postmortem [Patients were placed in a cold (4°C) room within 2 h after death] at the Brain Bank at Tokyo Metropolitan Institute of Gerontology. For all the brains registered at the bank we obtained written informed consents for their use for medical research from patient or patient's family. Each brain specimen (∼0.5 g) were taken from Brodmann areas 9–11 of 13 AD patients [80 ± 5.0 years of age, Braak NFT stage > IV, SP stage = C (retrospective) CDR ≫ 1], 10 MCI patients (91 ± 4.9 years of age, Braak NFT stage < IV, SP stage < C, CDR = 0.5) and 16 controls (77 ± 6.5 years of age, Braak NFT stage < I, SP stage = 0/A, CDR = 0) (Adachi et al, 2010; Li et al, 1997).

Cerebrospinal fluid analysis

Cerebrospinal fluid (10–15 ml) was collected in a polypropylene or polystyrene tube and gently inverted. After brief centrifugation CSF was aliquotized to polypropylene tubes (0.25–0.5 ml), which were kept at −80°C until use. In our experience, Aβ42 (possibly, other Aβ species too) are readily absorbed even to polypropylene tubes (∼20% per new exposure, as shown by Luminex xMAP quantification), and repeated aliquotization to new tubes may cause profoundly lower measures of Aβs (Tsukie and Kuwano, unpublished data, 2010). This may partly explain why absolute levels of Aβs in CSF greatly vary among laboratories, whereas their relative ratios (*e.g.* Aβ42/40) seem to be roughly consistent. The CSF concentrations of Aβ38, 40 and 42 were quantified using commercially available ELISA kits (Cat no. 27717, 27718 and 27712, respectively, IBL, Gunma, Japan). To measure Aβ43, anti-Aβ43 polyclonal antibody as a capture antibody was combined with amino terminus-specific antibody (82E1) (Cat no. 10323, IBL, Gunma, Japan) as a detector antibody. The detection limit of Aβ43 quantified by the ELISA was 0.78 pM (data not shown). Thus all ELISAs used here detect Aβ1-x, but not amino-terminally truncated Aβs. The specificities of ELISAs are provided in Supporting Information Fig S1.

CSF immunoprecipitation and Western blotting

When required, CSF Aβs were immunoprecipitated with protein G-sepharose conjugated with 82E1 at 4°C by keeping a container in gentle rotation overnight. The mixture was centrifuged at 10,000×g for 5 min, and resultant pellets were then washed twice with phosphate-buffered saline. The washed beads were suspended with the Laemmli sample buffer for SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The immunoprecipitated Aβs were separated on Tris/Tricine/8 M urea gels (Kakuda et al, 2006), followed by Western blotting using 82E1. To immunodetect Aβ42 and Aβ43, Aβ42 monoclonal antibody (44A3, IBL) and Aβ43 polyclonal antibody (IBL) were used (Supporting Information Fig S3).

Numerical simulation based on the stepwise processing model of γ-secretase

The temporal profiles for the ratios of Aβ40/43 and Aβ38/42 were simulated based on the stepwise processing model. Parameters including rate constants were set to fit maximally the temporal profile of the cleaving activity in the reconstituted γ-secretase system (Takami et al, 2009).

We set the condition that βCTF substrate is supplied steadily from the external source. When βCTF supply is balanced roughly in the order with γ-secretase processing rate, the stepwise-processing model was found to have the two successive steady states, with each accompanying linear changes in [ES] or [S] concentrations. The first steady state is just after the initial transition period that corresponds to the acute saturation phase of γ-secretase with βCTF. The second steady state is associated with the constant concentrations of the enzyme/substrate complex except ES38 and ES40. Because these steady states kept the ratios of Aβ38/Aβ40 and Aβ42/Aβ43 constant, the simulation was quite consistent with the CSF data.

Quantification of human brain raft-associated γ-secretase activity

Since γ-secretase is thought to be concentrated in rafts (Hur et al, 2008; Wada et al, 2003), we measured raft-associated γ-secretase activity rather than CHAPSO-solubilized activity. Rafts were prepared from human brains which were frozen within 12 h postmortem, as previously described (Oshima et al, 2001; Wada et al, 2003) with some modifications. We do not know exactly whether the γ-secretase activity depends upon the sampling site. In our hands, there appear no large differences in the activity among the sampled sites in a given prefrontal slice. No significant differences in the activity were noted between outer and inner layer of the cortex. After carefully removing leptomeninges and blood vessels, small (<0.5 g) blocks from prefrontal cortices (Brodmann areas 9–11) were homogenized in ∼10 volumes of 10% sucrose in MES-buffered saline (25 mM MES, pH 6.5, and 150 mM NaCl) containing 1% CHAPSO and various protease inhibitors. The homogenate was adjusted to 40% sucrose by the addition of an equal volume of 70% sucrose in MES-buffered saline, placed at the bottom of an ultracentrifuge tube, and overlaid with 4 ml of 35% sucrose and finally with 4 ml of 5% sucrose in MES-buffered saline. The discontinuous gradient was centrifuged at 39,000 rpm for 20 h at 4°C on a SW 41 Ti rotor (Beckman, Palo

The paper explained

PROBLEM:

Alzheimer's disease is a devastating form of progressive dementia, in which senile plaques composed of Aβ form in the brain. Different species of Aβ are derived from APP through sequential cleavage by β- and γ-secretases and can be detected in the CSF of patients. These can serve as markers for the disease.

RESULTS:

We investigated why CSF concentrations of Aβ42 are lower in MCI and AD patients. We suggest that this is not because Aβ42/43 is selectively deposited in the brain, but because γ-secretase activity is altered in AD brain: more Aβ42 and Aβ43 are converted to Aβ40 and Aβ38, respectively, resulting in lower Aβ42 and Aβ43 in CSF.

IMPACT:

Our results predict that γ-secretase modulators would have only limited efficacy in treatment of AD patients, because Aβ42/43 production by γ-secretase is already shifted towards reduced levels in AD brain.

Alto, CA). An interface of 5%/35% sucrose (fraction 2) was carefully collected (referred to as raft fraction). Raft fractions were recentrifuged after dilution with buffer C (20 mM PIPES, pH 7.0, 250 mM sucrose and 1 mM EGTA). The resultant pellet was washed twice and resuspended with buffer C, which was kept at −80°C until use.

As the method of measuring the raft γ-secretase activity was not yet established, we first determined the assay conditions. The incubation of raft fraction with βCTF generated exactly the same tri- and tetrapeptides we previously observed in the detergent-soluble γ-secretase assay system (Takami et al, unpublished observation). This suggests that the cleavage by raft-associated γ-secretase proceeds in the identical manner as by CHAPSO-reconstituted γ-secretase (Takami et al, 2009). In our hands, preexisting βCTF bound in rafts generated only negligible amounts of Aβs, and their generation was dependent exclusively on exogenously added βCTF. Thus, we concluded that the addition of βCTF to raft fraction make possible to measure the raft-associated γ-secretase activity, although we do not know how the exogenously added βCTF is integrated into raft, gets access to and is degraded by raft-embedded γ-secretase. Using this assay method, the activities of raft-associated γ-secretase in human brains were found to be only a little affected postmortem, when compared with that prepared from fresh rat brains. A progressive decline in the activity was barely detectable from 4 to 17 h postmortem. The discrepancy in the postmortem decay between our and the previous data (Hur et al, 2008) would be ascribed to the assay method: the latter are based on the activity measured by using endogenous (raft-bound) substrate that is also susceptible to proteolytic degradation (Hur et al, 2008).

Each raft fraction, adjusted to 100 μg/ml in protein concentration, was incubated with 200 nM C99FLAG for 2 h at 37°C (Kakuda et al, 2006). The produced Aβs were separated on SDS–PAGE, and subjected to quantitative Western blotting, using specific antibodies, 3B1 for Aβ38, BA27 for Aβ40, 44A3 for Aβ42 and anti-Aβ43 polyclonal for Aβ43.

Statistical analysis

All statistical analyses were performed using SPSS version 14.0. The results were expressed as means ± standard deviations. Because data transformations were required to achieve normally distributed data, all analyses including Aβ38, Aβ40, Aβ42 and Aβ43 were performed after a logarithmic transformation. Pearson's correlation coefficients were calculated to indicate the strength of the linear relationship between two variables. An ANOVA was used to test the equality of mean values of continuous variables among three groups, that is control, MCI and AD. Multiple comparisons were done by Dunnett's *t*-test, Bonferroni's *t*-test and Welch's *t*-test between control and MCI/AD, and among three groups, respectively. A two-tailed *p*-value of <0.05 was considered to be statistically significant.

Author contributions

NK, MT, KN, YI: measurement of raft-associated γ-secretase activity in human brains, LC-MS/MS confirmation of released peptides, ELISA quantification of Aβ38, 40, 42 and 43 in CSF and tissue blocks, and experimental design of the present work; MS, HiA, KF, TI, and the Japanese Alzheimer's Disease Neuroimaging Initiative: collection of CSF samples from controls, MCI/AD patients; YH, MM, HaA: collection of CSF from iNPH patients; HY, SM, HH: Aβ immunocytochemistry of tissue sections from brains with various SP stages (Braak); KA: statistical analysis; RK: establishment of the appropriate Aβ quantification conditions; YN: simulation of the stepwise processing model.

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Supporting Information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

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