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Dominant and Differential Deposition of Distinct β-Amyloid Peptide Species, $A\beta_{N3(pE)}$, in Senile Plaques

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Summary

We analyzed an amino-terminal modification of β-amyloid (Aβ) peptide in brain, using anti-Aβ antibodies that distinguish distinct molecular species. Examination of cortical sections from 28 aged individuals with a wide range in senile plaque density revealed that a molecular species distinct from the standard Aβ is deposited in the brain in a dominant and differential manner. This modified Aβ peptide ($A\beta_{N3(pE)}$) starts at the 3rd amino-terminal residue of the standard Aβ, glutamate, converted to pyroglutamate through intramolecular dehydration. Because plaques composed of $A\beta_{N3(pE)}$ are present in equivalent or greater densities than those composed of standard Aβ bearing the first amino-terminal residue ($A\beta_{N1}$) and because deposition of the former species appears to precede deposition of the latter, as confirmed with specimens from Down's syndrome patients, the processes involved in $A\beta_{N3(pE)}$ production and retention may play an early and critical role in senile plaque formation.

Introduction

The correlation between the progressive deposition of β-amyloid (Aβ) peptides in senile plaques in brain and the pathological onset of Alzheimer's disease (AD) indicates that Aβ metabolism is involved in the disease development as a critical factor (Selkoe, 1993; Mullan and Crawford, 1993). Therefore, to clarify how the peptides are processed during the course of senile plaque formation and how their structures affect their pathogenic properties is an essential step toward understanding the pathological mechanism of AD. Because a subtle difference of 2 amino acid residues in the Aβ carboxy-terminal sequence drastically alters their physicochemical parameters linked to plaque formation (Jarrett and Lansbury, 1993), caution is now required so as not to overlook any in vivo modifications that might potentially affect the Aβ pathogenicity. While information on carboxy-terminal variations and their roles is accumulating (Miller et al., 1993; Suzuki et al., 1994; Iwatsubo et al., 1994), little is as yet known about modifications at the other end of the molecule, owing in part to technical obstacles.

A number of reports have described the structures of Aβ peptides isolated from AD brains (Glenner and Wong, 1984; Masters et al., 1985; Selkoe et al., 1986; Mori et al., 1992; Roher et al., 1993a, 1993b; Miller et al., 1993; Gowing et al., 1994). Although discrepancies exist depending on the purification procedures and analytical methods employed, we were particularly interested in the study by Mori et al. (1992) describing the consistent (15%–20%) presence of an Aβ peptide bearing an amino-terminal pyroglutamate ($A\beta_{N3(pE)}$), in addition to the major species bearing the standard initial amino terminus ($A\beta_{N1}$). Because the amino terminus of the $A\beta_{N3(pE)}$ peptide is blocked by internal lactam (see Figure 1A), it is protected from the proteolytic action of aminopeptidases other than pyroglutamate-specific ones (McDonald and Barrett, 1986) and can thus remain stable in tissues. The peptide is also resistant to Edman degradation and is thus usually overlooked in chemical sequencing. Furthermore, the $A\beta_{N3(pE)}$ peptide is also likely to possess altered physicochemical characteristics because the conversion of $A\beta_{N1}$ to $A\beta_{N3(pE)}$ results in the loss of one positive and two negative charges per molecule. These unique properties of $A\beta_{N3(pE)}$ led us to assume that a possible involvement of this amino-terminal modification in amyloid plaque formation had not yet been examined fully. We have therefore conducted an in situ analysis of this amino-terminal modification so that the results might not be influenced by purification procedures and that spatial information concerning the localization of $A\beta_{N3(pE)}$ as compared with $A\beta_{N1}$ might be obtained.

Results

Antibodies Specific to Distinct Amino-Terminal Structures of Aβ

As a tool to distinguish between $A\beta_{N1}$ and $A\beta_{N3(pE)}$, shown in Figure 1A, we developed antibodies of novel characteristics: anti-$A\beta_{1-5}$ for the former (Saido et al., 1994a) and anti-$A\beta_{3(pE)-7}$ for the latter. The unique methodology used to produce such proteolytic product-specific antibodies was developed in our previous studies (Saido et al., 1992, 1993, 1994b). Figures 1B and 1C show the specificity of these antibodies and another anti-Aβ antibody, anti-$A\beta_{1-28}$ (Kanemaru et al., 1990). In Western blot analysis, the anti-

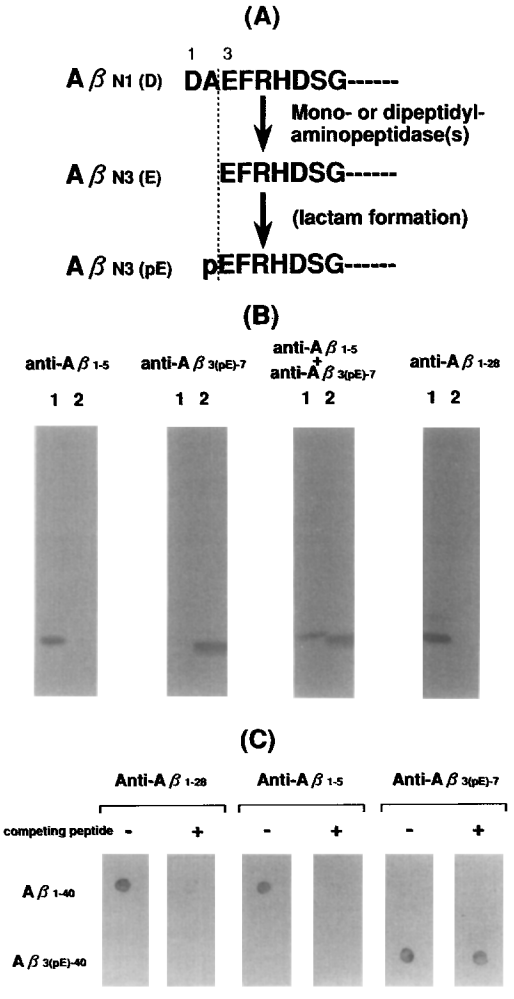

Figure 1. Amino-Terminal Modification of Aβ Peptides and Modification-Specific Anti-Aβ Antibodies

(A) The amino-terminal aspartylalanyl moiety of the standard $Aβ_{N1}$ peptide is removed by mono- or dipeptidylaminopeptidase(s) to yield a new terminal glutamate residue, which readily converts to the pyro-form ($Aβ_{N3(pE)}$) through intramolecular dehydration. Note that this modification results in the loss of one positive and two negative charges. The product is unique in being resistant to most aminopeptidases and to chemical sequencing. pE, pyroglutamate.

(B) Synthetic peptides (50 ng) corresponding to $Aβ_{1-40}$ (lane 1) and $Aβ_{3(pE)-40}$ (lane 2) were subjected to Western blot analysis on 5%–20% gradient gel using the indicated antibodies.

(C) The $Aβ_{1-40}$ and $Aβ_{3(pE)-40}$ peptides (20 ng) were subjected to dot blot analysis using the anti-Aβ antibodies as indicated. Antibody binding was performed in the absence (−) or presence (+) of the antigenic hapten peptide for the anti-$Aβ_{1-5}$ antibody, DAEFRC (40 μM), as previously described (Saido et al., 1994a).

$Aβ_{1-5}$ and anti-$Aβ_{3(pE)-7}$ antibodies recognized $Aβ_{1-40}$ and $Aβ_{3(pE)-40}$ peptides, respectively, in a manner strictly exclusive of each other. The primary structures of the synthetic peptides were verified by mass spectroscopy (electron spray) and peptide mapping. These results were confirmed further by dot blot analysis (Figure 1C), in which binding of anti-$Aβ_{1-5}$ to the $Aβ_{1-40}$ peptide was blocked by the $Aβ_{1-5}$ peptide, whereas the binding of anti-$Aβ_{3(pE)-7}$ to the $Aβ_{3(pE)-40}$ peptide was not.

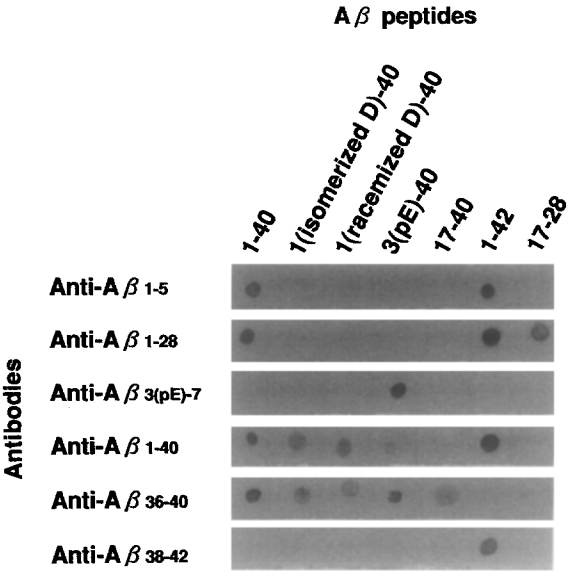

Figure 2. Reactivity of Various Anti-Aβ Antibodies with Standard and Modified Aβ Peptides

The standard and modified synthetic Aβ peptides—1-40, 1(isomerized D)-40, 1(racemized D)-40, 3(pE)-40, 17-40, 1-42, and 17-28—were subjected to dot blot analysis using the indicated antibodies as described in the legend to Figure 1C. The labels 1(isomerized D)-40 and 1(racemized D)-40 stand for $Aβ_{1-40}$ peptides possessing isoaspartate and racemized aspartate at the amino terminus, respectively.

The anti-$Aβ_{1-28}$ antibody was originally used as a positive control antibody that would recognize both $Aβ_{1-40}$ and $Aβ_{3(pE)-40}$ peptides because of the larger epitope range of Aβ that it was expected to cover. Unexpectedly, this antibody bound only to $Aβ_{1-40}$ and completely failed to recognize $Aβ_{3(pE)-40}$ (Figures 1B and 1C), suggesting that conformational restriction may exist in the interaction between the antibody and Aβ molecules. The anti-$Aβ_{1-28}$ and anti-$Aβ_{1-5}$ antibodies seem to share similar amino-terminal specificities because binding of both antibodies to $Aβ_{1-40}$ was fully inhibited by the $Aβ_{1-5}$ peptide, but not by $Aβ_{3(pE)-7}$.

The specificity of these antibodies was examined further as compared with other anti-Aβ antibodies using various modified synthetic Aβ peptides that have been shown to be present in AD brains (Figure 2). Surprisingly, neither the anti-$Aβ_{1-5}$, the anti-$Aβ_{3(pE)-7}$, nor the anti-$Aβ_{1-28}$ antibody reacted with the $Aβ_{1-40}$ peptides bearing isomerized or racemized aspartate at the amino terminus. The anti-$Aβ_{1-28}$ antibody differed from anti-$Aβ_{1-5}$ in that only this antibody recognized the $Aβ_{17-28}$ peptide. Because the anti-$Aβ_{1-28}$ antibody failed to bind to the $Aβ_{17-40}$ peptide, we may conclude that this antibody also recognizes the carboxyl terminus of the $Aβ_{x-28}$ peptide. The binding of anti-$Aβ_{1-5}$ and anti-$Aβ_{1-28}$ antibodies to Aβ peptides does not seem to be influenced by the carboxy-terminal structure of the peptides, as both also recognized the $Aβ_{1-42}$ peptide.

The anti-$Aβ_{1-40}$ antibody bound strongly to the $Aβ_{1-40}$ and $Aβ_{1-42}$ peptides and more weakly to the others with varying degrees; the $Aβ_{3(pE)-40}$ peptide was barely recognized by this antibody. It should also be noted that the reactivity

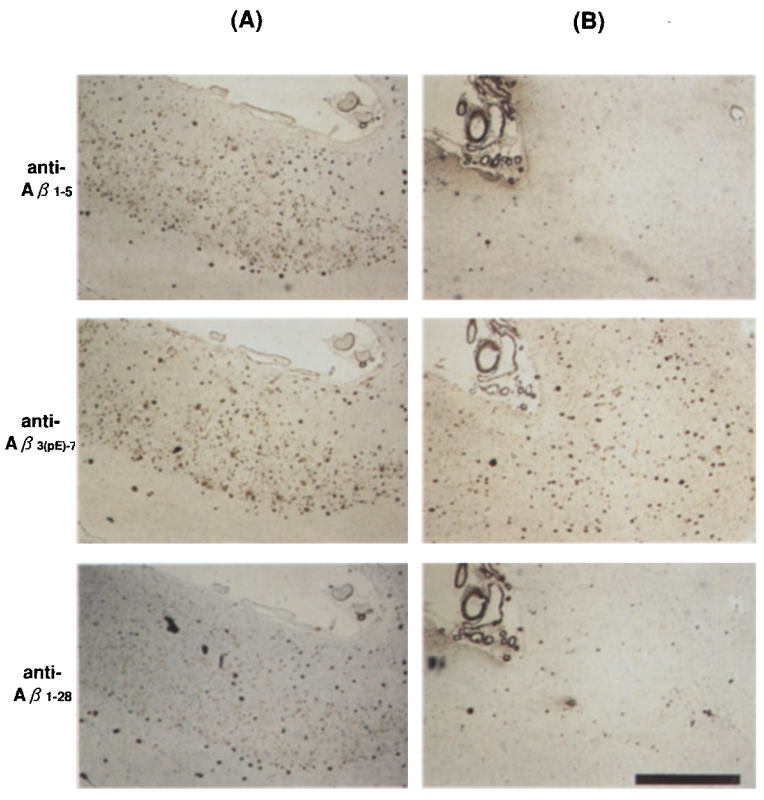

Figure 3. Deposition of Distinct Aβ Species in Aged Brains

Sets of serial cerebral sections (6 μm thick) from 28 postmortem brains were immunostained using anti-$A\beta_{1-5}$, anti-$A\beta_{3(pE)-7}$, and anti-$A\beta_{1-28}$ antibodies as indicated. (A) and (B) show representative sections taken from different individuals. See the text for details. Bar, 1 mm.

of antisera raised against such a relatively large peptide bearing multiple possible epitopes is likely to vary depending on lots of the sera. The anti-$A\beta_{36-40}$ antibody bound exclusively to Aβ peptides ending with the 40th residue at the carboxyl terminus, whereas the anti-$A\beta_{38-42}$ antibody bound only to $A\beta_{1-42}$.

These data not only confirm the specificities of the anti-$A\beta_{1-5}$ and anti-$A\beta_{3(pE)-7}$ antibodies toward their target molecules but also indicate that few anti-Aβ antibodies react evenly with various forms of Aβ molecules in a strict sense. Terminal structures of the peptide seem to have profound influence on its binding of antibodies. This is probably because the antigenicity of synthetic Aβ is heavier at the termini than other portions of the molecule. Consequently, the way Aβ peptides appear to us depends heavily on what kind of antibody we use to visualize them. We must therefore bear in mind that it is not easy to detect total Aβ peptides impartially by immunochemical means and that use of anti-Aβ antibodies with a narrow epitope range could result in negligence of certain forms of Aβ peptides. It is thus critical to employ well-defined antibodies for detecting specific Aβ peptides in human brain tissues.

Immunohistochemistry: Dominant Deposition of the $A\beta_{N3(pE)}$ Peptide in Senile Plaques

Using this set of anti-Aβ antibodies, we performed immunohistochemical observations on serial sections from cerebral cortex so that their staining patterns could be compared directly (Figure 3). The postmortem brains used were from nondemented and demented individuals ranging in age from 68 to 99 years (average, 84.7; standard deviation, 10.0) and showing various degrees of amyloid deposition. Results can be divided into two major groups according to the relative density of the immunoreactive plaques. In one, approximately as many plaques were immunostained by anti-$A\beta_{1-5}$ ($A\beta_{N1}$ plaques) or anti-$A\beta_{1-28}$ as were stained by anti-$A\beta_{3(pE)-7}$ ($A\beta_{N3(pE)}$ plaques; Figure 3A). In the other, the $A\beta_{N3(pE)}$ plaques markedly exceeded the $A\beta_{N1}$ plaques in density (Figure 3B). In only a few cases, the $A\beta_{N1}$ plaques slightly exceeded the $A\beta_{N3(pE)}$ plaques (see below). The anti-$A\beta_{1-5}$ and anti-$A\beta_{1-28}$ antibodies showed similar staining patterns in accordance with their shared binding specificity toward the amino terminus of Aβ (Figure 1). Other lots of anti-$A\beta_{1-5}$ and anti-$A\beta_{3(pE)-7}$ antibodies raised in different rabbits gave similar immunostaining patterns.

To quantitate these observations, we counted the number of plaques immunostained by each antibody in the cortical sections and plotted them against each other (Figure 4). As shown in Figure 4A, the straightforward positive correlation in density between plaques immunostained by anti-$A\beta_{1-5}$ and by anti-$A\beta_{1-28}$ (correlation coefficient, $r = 0.956$) emphasizes the immunoreactive similarities of these antibodies. Figure 4B compares the densities of plaques immunostained by anti-$A\beta_{1-5}$ and by anti-$A\beta_{3(pE)-7}$, showing a much weaker correlation ($r = 0.624$). In this case, most of the data points are located around or above the 1:1 ratio line, substantiating the notion that $A\beta_{N3(pE)}$ plaques are present in equivalent or greater densities than $A\beta_{N1}$ plaques.

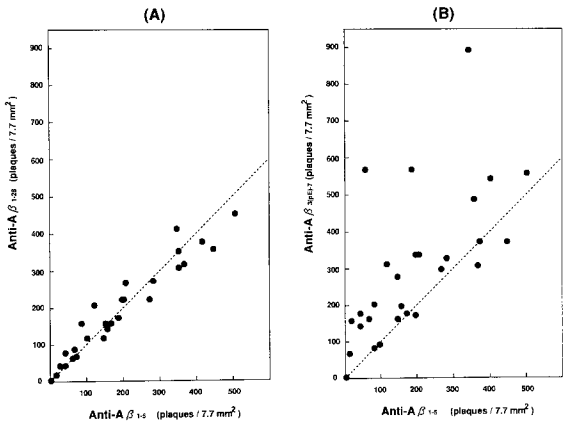

Figure 4. Correlation in Density among Aβ Plaques Immunostained with Anti-$A\beta_{1-5}$, Anti-$A\beta_{1-28}$, and Anti-$A\beta_{3(pE)-7}$ Antibodies

Sets of immunostained serial sections from 28 brains were photographed, and the numbers of only distinct plaques, both the diffuse and compact types, were counted.

(A) Correlation in density of plaques stained with anti-$A\beta_{1-28}$ and those stained with anti-$A\beta_{1-5}$.

(B) Density of anti-$A\beta_{3(pE)-7}$-reactive versus anti-$A\beta_{1-5}$-reactive plaques. The dotted lines indicate the points at which the densities of the plaques being compared equal each other.

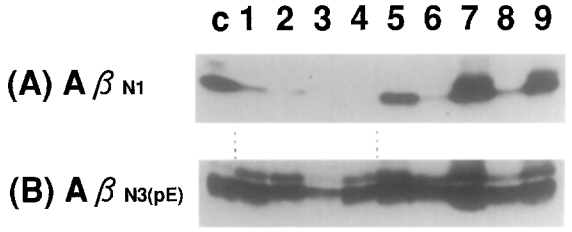

Figure 5. Western Blot Analysis of $A\beta_{N1}$ and $A\beta_{N3(pE)}$ Peptides in Formic Acid Extracts of Brain

Aβ peptides were extracted by formic acid from frozen brain samples available for the analysis and subjected to Western blot analysis using the anti-$A\beta_{1-5}$ (A) and anti-$A\beta_{3(pE)-7}$ (B) antibodies. Lane "c" contains 100 ng of the corresponding control synthetic peptides, $A\beta_{1-40}$ (A) and $A\beta_{3(pE)-40}$ (B). Lanes 1–4 represent the samples from brains with $A\beta_{N3(pE)}$ plaques being immunohistochemically dominant (plaque ratio of $A\beta_{N3(pE)}$ to $A\beta_{N1} > 1.5$), while lanes 5–9 show samples from brains in which $A\beta_{N1}$ and $A\beta_{N3(pE)}$ plaques appeared comparable in density (plaque ratio < 1.5). Note that these samples were not necessarily taken from exactly the same brain areas as those used for immunohistochemistry. Three separate experiments were performed to confirm and quantitate the shown data (see Table 1).

Biochemical Analysis of $A\beta_{N1}$ and $A\beta_{N3(pE)}$ Peptides in Brain

To obtain a biochemical basis for the immunohistochemical observation that the $A\beta_{N3(pE)}$ plaques are present in brain in predominant or equivalent density as compared with the $A\beta_{N1}$ plaques (Figure 3; Figure 4), we performed Western blot analyses to quantitate these peptides extracted by formic acid from brains (Figure 5; Table 1). We did not employ the method using high pressure liquid chromatography and enzyme-linked immunosorbent assays (Suzuki et al., 1994), because $A\beta_{N3(pE)}$ peptides showed particularly poor recovery in reverse-phase chromatography and low solubility in an aqueous-phase chromatography (data not shown). The samples represent the brains in which $A\beta_{N3(pE)}$ plaques are predominant (lanes 1–4) and those in which $A\beta_{N1}$ and $A\beta_{N3(pE)}$ plaques are comparable (lanes 5–9) as immunohistochemically analyzed. Specificity of the antibody binding was confirmed by competition experiments as in Figure 1.

The amount of $A\beta_{N1}$ varied from less than 0.05 to 30.5 micrograms per gram of wet brain, whereas the amount of $A\beta_{N3(pE)}$ varied from 5.6 to 91.0 micrograms per gram. Surprisingly, the latter was greater than the former in all the brains examined, indicating fairly ubiquitous presence of this amino-terminal modified form of Aβ. These data also imply that the amount of $A\beta_{N3(pE)}$ peptide per plaque may be greater than that of $A\beta_{N1}$ peptide. Furthermore, the results exclude the possibility that the immunohistochemical visualization may be more sensitive for detecting $A\beta_{N3(pE)}$ than for $A\beta_{N1}$, which could have resulted in overestimation of $A\beta_{N3(pE)}$ plaques.

It is also clear that the amount of $A\beta_{N3(pE)}$ relative to $A\beta_{N1}$ was significantly greater in the immunohistochemically $A\beta_{N3(pE)}$ plaque–dominant brains than in the brains in which $A\beta_{N1}$ and $A\beta_{N3(pE)}$ plaques were present in comparable densities. These results agree with the immunohistochemical observation showing the dominant deposition of $A\beta_{N3(pE)}$ peptides in these brains. We must, however, point out that caution is required in interpreting these biochemical data, because efficiency of extraction may vary depending on the peptide species and because spatial information concerning the localization of the peptides (i.e., plaque amyloid versus vascular amyloid) is lost by homogenization.

Ratio Analysis of Immunohistochemical Data: Implications for Chronological Relationship between the $A\beta_{N1}$ and $A\beta_{N3(pE)}$ Plaques

We further analyzed the immunohistochemical data to investigate whether $A\beta_{N1}$ and $A\beta_{N3(pE)}$ plaques are evolutionally related to each other. Figure 6 shows the relations between the density of $A\beta_{N1}$ or $A\beta_{N3(pE)}$ plaques and the ratio of $A\beta_{N3(pE)}$ plaques to $A\beta_{N1}$ plaques. The logical basis for this analysis lies in the assumption that each individual brain represents a various developmental stage of Aβ deposition, giving clues to resolving the processes in chrono-

Table 1. Biochemical Quantitation of $A\beta_{N1}$ and $A\beta_{N3(pE)}$ Peptides Extracted from Brains

Number	Peptide (μg/g wet brain) $A\beta_{N1}$	Peptide (μg/g wet brain) $A\beta_{N3(pE)}$	Ratio ($A\beta_{N3(pE)}/A\beta_{N1}$)
$A\beta_{N3(pE)}$ plaque–dominant brains			
1	3.2	35.0	10.9
2	1.4	24.0	17.1
3	>0.05	5.6	>112.0
4	0.23	19.0	82.0
$A\beta_{N1}$ and $A\beta_{N3(pE)}$ plaque–comparable brains			
5	8.4	59.0	7.0
6	2.1	17.0	8.0
7	30.5	91.0	3.0
8	3.0	17.0	5.7
9	14.5	43.0	3.0

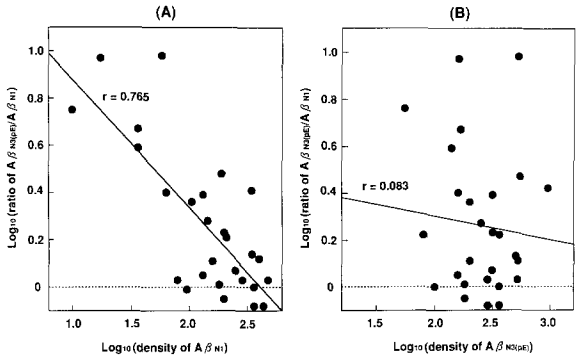

Figure 6. Ratio Analysis of $A\beta_{N1}$ and $A\beta_{N3(pE)}$ Plaques

Ratios of $A\beta_{N3(pE)}$ plaques to $A\beta_{N1}$ plaques were calculated from the data shown in Figure 3B and plotted against the density of $A\beta_{N1}$ plaques (A) or the density of $A\beta_{N3(pE)}$ plaques (B) on logarithmic scales. Linear regression analyses were performed to acquire the regression lines. The dotted lines indicate ratios of 1. r, correlation coefficient.

Table 2. Density of $A\beta_{N1}$ and $A\beta_{N3(pE)}$ Plaques in Brains from Down's Syndrome Patients of Various Ages

Age/Sex	Plaque Density (per 7.7 mm^2)[a] $A\beta_{N1}$	$A\beta_{N3(pE)}$	Ratio ($A\beta_{N1}/A\beta_{N3(pE)}$)
31/M	0	6	0.00
37/F	4	52	0.08
38/F	23	173	0.13
41/F	21	56	0.38
42/F	80	161	0.50
44/M	34	78	0.44
53/M	24	25	0.96
56/M	201	260	0.77
57/M	328	241	1.36
58/F	211	739	0.29
59/M	50	126	0.40
59/M	186	302	0.61
62/F	484	332	1.46
64/M	173	336	0.51

[a] The numbers of plaques were counted as described in the legend to Figure 4.

logical terms. The ratios and the densities of $A\beta_{N1}$ plaques are apparently well correlated (r = 0.765) on logarithmic scales; the ratios decreased and converged to 1:1 as plaque densities increased (Figure 6A). In contrast, no such tendency is apparent between the ratio and the density of $A\beta_{N3(pE)}$ plaques (Figure 6B). These data suggest that $A\beta_{N1}$ plaque formation was influenced by the ratio of $A\beta_{N3(pE)}$ to $A\beta_{N1}$ plaques, whereas the $A\beta_{N3(pE)}$ plaques arose in an independent manner. The most relevant interpretation of this relationship in statistical terms would be that $A\beta_{N1}$ deposition followed $A\beta_{N3(pE)}$ deposition in these brains. This possibility was examined further by comparing the plaque patterns in different cerebral cortical areas taken from the same individuals.

$A\beta_{N1}$ versus $A\beta_{N3(pE)}$ in Different Areas in Brain

We examined 22 brains for which complete sets of frontal, temporal, and occipital sections were available for comparison. In 4 cases, there was a clearly different relative plaque distribution depending on the brain area (Figure 7). In 3 of these brains, the density of $A\beta_{N3(pE)}$ plaques evidently exceeded the density of $A\beta_{N1}$ plaques in frontal and temporal lobes, whereas they were present in comparable densities in the occipital lobes. There was also 1 case in which the $A\beta_{N3(pE)}$ to $A\beta_{N1}$ ratio was greater in the occipital and temporal lobes than in the frontal lobe. There were 7 other cases that showed weaker but distinct deviation in the relative immunoreactivity among the brain areas.

Because the evolution of amyloid plaques may proceed at different rates within these brain regions before reaching overall saturation (Braak and Braak, 1991; Armstrong et al., 1993), these observations favor the assumption that $A\beta_{N3(pE)}$ deposition precedes $A\beta_{N1}$ deposition in the course of plaque development. Furthermore, the anti-$A\beta_{3(pE)-7}$ antibody reacted with diffuse plaques far more intensely than the anti-$A\beta_{1-5}$ antibody (compare the lower panels). This again indicates the presence of $A\beta_{N3(pE)}$ peptide, rather than $A\beta_{N1}$, in early phases of amyloid deposition, since diffuse plaques represent an initial stage of senile plaque formation (Yamaguchi et al., 1989).

$A\beta_{N1}$ versus $A\beta_{N3(pE)}$ in Brains from Down's Syndrome Patients

To examine further the above assumption concerning the chronological relationship between these Aβ species under conditions allowing a more straightforward interpretation, we immunostained brain sections from Down's syndrome patients of various ages. This is based on the knowledge that the extent of amyloid plaque formation relates far better to age in Down's syndrome than in normal aging and sporadic AD (Wisniewski et al., 1985; Oliver and Holland, 1986), even though the underlying mechanism for Aβ deposition may not necessarily be identical in a strict sense.

Table 2 and Figure 8 summarize the immunohistochemical observations of 14 Down's syndrome cases using the anti-$A\beta_{1-5}$ and anti-$A\beta_{3(pE)-7}$ antibodies. Most younger subjects under 50 years of age (Figure 8A) displayed very few anti-$A\beta_{1-5}$-positive plaques. Even in these cases, the anti-$A\beta_{3(pE)-7}$-positive plaques were clearly present in greater densities. Older subjects, i.e., over 50 years of age (Figure 8B), provided more variable results, with $A\beta_{N3(pE)}$ plaques being either equivalent or greater in density than $A\beta_{N1}$ plaques. Notably, the ratio of $A\beta_{N1}$ to $A\beta_{N3(pE)}$ correlated significantly with age (ratio = 0.026 × age − 0.75; r = 0.64). These data firmly support the assumption that deposition of $A\beta_{N3(pE)}$ precedes that of $A\beta_{N1}$. It should be noted, however, that $A\beta_{N3(pE)}$ may not necessarily represent the entire population of nonstandard Aβ molecules with modified amino termini present in these brains. Further studies focusing on other possible modifications (Roher et al., 1993a; Gowing et al., 1994) will be necessary to address this question.

Differential Deposition of $A\beta_{N1}$ and $A\beta_{N3(pE)}$ Peptides in Senile Plaques

Finally, we investigated whether these Aβ molecules dif-

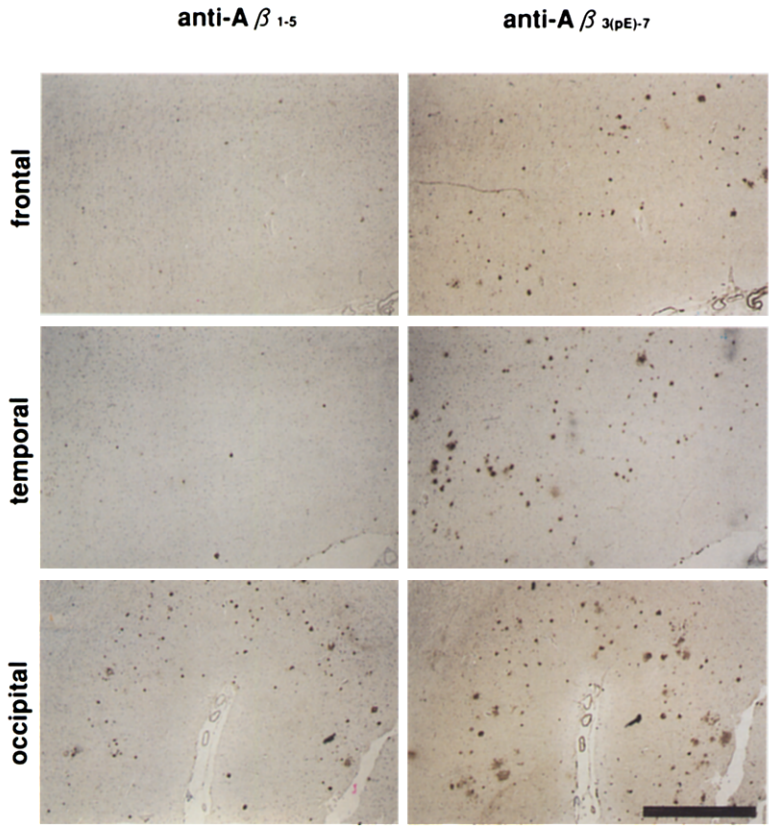

Figure 7. Varying Relative Deposition of Aβ Species, $A\beta_{N1}$ and $A\beta_{N3(pE)}$, in Different Cortical Areas from an Individual Brain

Cerebral sections taken from the frontal, temporal, and occipital lobes of a brain were immunostained with anti-$A\beta_{1-5}$ and anti-$A\beta_{3(pE)-7}$ antibodies. Shown is 1 of the 4 brains within which the relative densities of the two plaque types clearly varied depending on the cerebral areas. See the text for details. Bar, 1 mm.

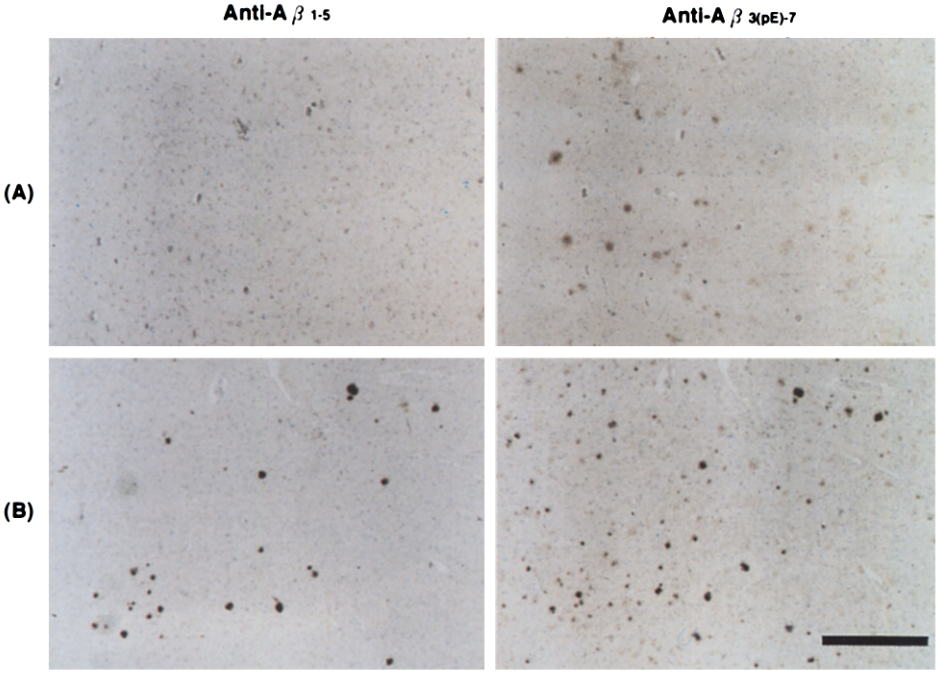

Figure 8. $A\beta_{N1}$ and $A\beta_{N3(pE)}$ Plaques in Down's Syndrome Patients

Serial sections from brains of Down's syndrome patients were immunostained with anti-$A\beta_{1-5}$ and anti-$A\beta_{3(pE)-7}$ antibodies as in Figure 3. (A) and (B) show representative sections from young (38-year-old female) and old (58-year-old female) subjects, respectively. Bar, 0.5 mm.

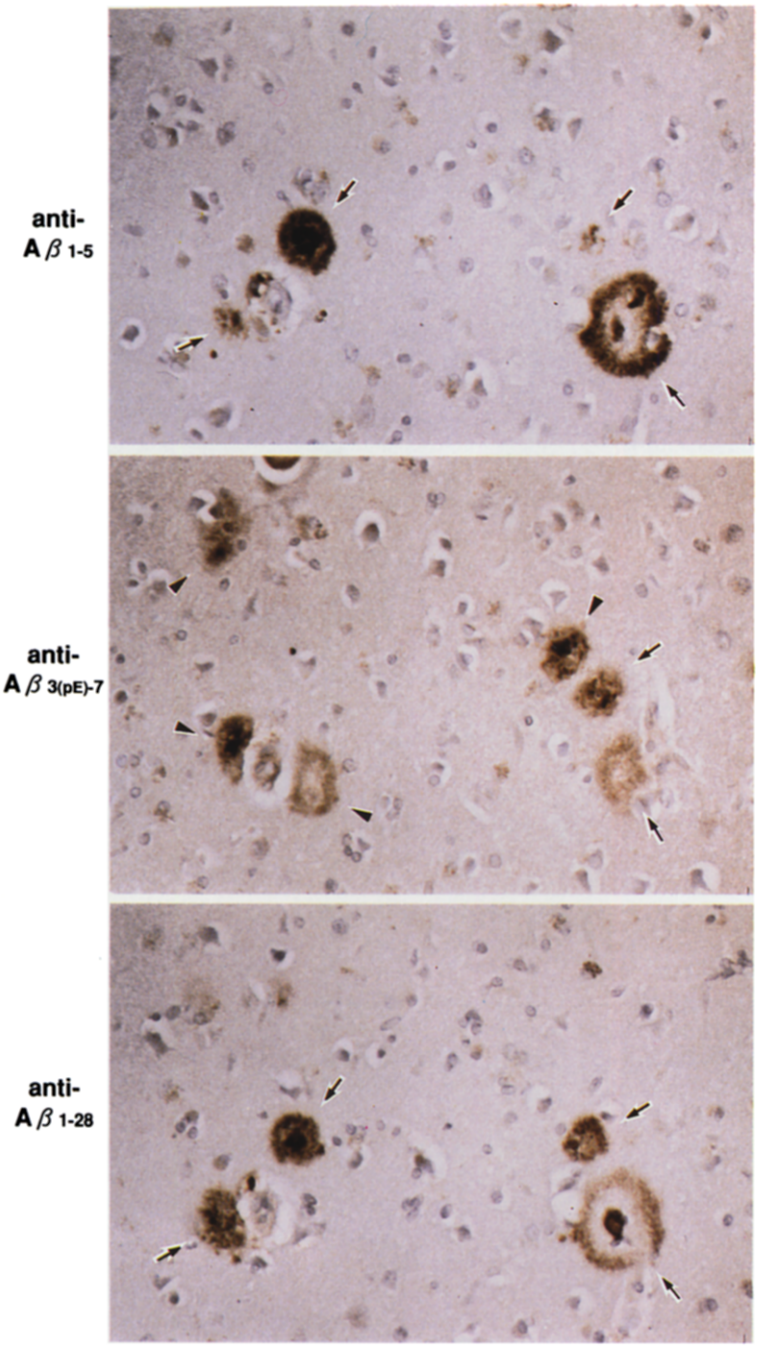

Figure 9. Differential Deposition of $A\beta_{N1}$ and $A\beta_{N3(pE)}$ Peptides in Individual Plaques

A set of serial sections immunostained as in Figure 2 are shown at greater magnification. A cerebral area showing typical, relatively mild Aβ deposition (157 $A\beta_{N1}$ plaques and 277 $A\beta_{N3(pE)}$ plaques per 7.7 mm^2) was chosen. See the text for definitions of arrows and arrowheads. Bar, 100 μm.

fering in amino-terminal structure are present together or separately in individual senile plaques. Figure 9 shows serial sections immunostained with the three anti-Aβ antibodies, at a greater magnification than the previous figures. The anti-$A\beta_{1-5}$ and anti-$A\beta_{1-28}$ antibodies bound to common plaques (as indicated by the arrows in the top and bottom panels), agreeing with the data given in Figures 1–4. Plaques weakly stained only by the anti-$A\beta_{1-28}$ antibody (upper left portion of the bottom panel) may contain a nonstandard Aβ peptide with the carboxyl terminus of $A\beta_{x-28}$ (Figure 2). The anti-$A\beta_{3(pE)-7}$-stained section (middle panel), sandwiched between the other two, shows a distinct pattern. The plaques indicated by the arrowheads are anti-$A\beta_{3(pE)-7}$-positive and anti-$A\beta_{1-5}$-negative, while some of the plaques recognized by the anti-$A\beta_{1-5}$ and anti-$A\beta_{1-28}$ antibodies are not immunostained in this section. The other plaques, indicated by the arrows, seem to be composed of mixed Aβ peptides recognized by each of the antibodies. To summarize, some plaques consist exclusively of either $A\beta_{N1}$ or $A\beta_{N3(pE)}$, while others contain both. This observation, together with the data suggesting an earlier deposition of $A\beta_{N3(pE)}$ (Figures 3–8; Table 1; Table 2), indicates that conversion of $A\beta_{N1}$ to $A\beta_{N3(pE)}$ probably takes place prior to plaque formation, and that these differ-

ent molecular species of Aβ tend to aggregate through homophilic interactions, although they can also coaggregate. Once aggregated, the peptides will enter a solid state, under which any structural modification will proceed far more slowly than in solution.

Discussion

In the present study, we have demonstrated that a distinct Aβ peptide, $Aβ_{N3(pE)}$, is deposited in senile plaques in a dominant and differential manner as compared with the standard $Aβ_{N1}$ peptide. Both immunohistochemical and biochemical data (Figures 3–5; Table 1) support this observation. One advantage of our approach in detecting such a modified molecule in situ, as compared with the orthodox biochemical approaches, is that this approach minimizes any possible loss of the material that may otherwise result from peptide isolation procedures. Furthermore, the ratio analysis of the immunohistochemical data (Figure 6) and the regional difference of the Aβ deposition (Figure 7) led to the speculation that deposition of $Aβ_{N3(pE)}$ in senile plaques may have preceded that of $Aβ_{N1}$. This possibility was strengthened significantly by the observation that $Aβ_{N3(pE)}$ plaques appeared earlier than $Aβ_{N1}$ plaques in Down's syndrome brains (Figure 8; Table 2).

Our results suggest that amino-terminal variations in Aβ, in addition to the carboxy-terminal differences (Roher et al., 1993a, 1993b; Miller et al., 1993; Suzuki et al., 1994; Iwatsubo et al., 1994), must be taken into account when examining the pathological roles of Aβ peptides associated, for instance, with depositability (Jarrett and Lansbury, 1993) or neurotoxicity (Yankner et al., 1989; Mattson et al., 1993). We propose that $Aβ_{3(pE)-40}$ and $Aβ_{3(pE)-42}$ peptides as well as $Aβ_{1-40}$ and $Aβ_{1-42}$ peptides be the subject of such studies. It is quite possible that $Aβ_{3(pE)-42}$ is even more depositable than $Aβ_{1-42}$, because the former is expected to be more hydrophobic owing to the loss of one positive and two negative charges (Figure 1). It is also likely that $Aβ_{N3(pE)}$ has longer biological life in tissues than other Aβ peptides with free amino termini, since the latter can be digested by various aminopeptidases while the former is sensitive only to pyroglutamate-specific ones.

We should also not overlook other amino-terminal modifications of Aβ, such as racemization, isomerization, and limited proteolysis at other sites (Roher et al., 1993a; Gowing et al., 1994). In addition, heterogeneous Aβ peptides produced by cultured cells (Haass and Selkoe, 1993), particularly by neuronal cells, should be considered. We are currently attempting to produce antibodies that will distinguish these modified forms from one another and standard Aβ; they should be useful in elucidating the evolutionary relationships among Aβ molecules possessing various amino-terminal structures. It is also critically important to produce or identify an "almighty anti-Aβ antibody" that would impartially recognize any forms of Aβ, because all the antibodies used in the present study react differentially with various Aβ peptides (Figure 2). Accumulation of data based on both biochemical and immunohistochemical approaches should eventually lead to clarifying the Aβ processing associated with senile plaque formation.

Because of the lack of such almighty antibodies, we do not know exactly what portion of the entire Aβ population $Aβ_{N3(pE)}$ constitutes. However, its amount relative to the standard $Aβ_{N1}$ peptide is significantly large (Table 1), indicating that it is probably one of the major species. We speculate that the following factors associated with isolation, identification, and analysis of Aβ may influence biochemical quantitation of modified Aβ peptides. First, the efficiency of extraction may vary depending on the peptide species. Second, anti-Aβ antibodies recognizing narrow epitope ranges of the antigen will neglect certain forms of Aβ, as demonstrated in Figure 2. Third, reverse-phase chromatography of $Aβ_{N3(pE)}$ peptides, particularly $Aβ_{3(pE)-42}$, results in low recovery. Fourth, Aβ peptides possessing a blocked amino terminus, such as acetylated Aβ and $Aβ_{N3(pE)}$, are resistant to Edman degradation and thus are totally neglected in chemical sequence analyses, including radiosequencing. Fifth, tryptic digestion of $Aβ_{N3(pE)}$ peptides as a pretreatment for mass spectroscopic analysis results in the production of an amino-terminal fragment as short as a trimer, which may no longer be recovered in the subsequent reverse-phase chromatography. Isolation and characterization of Aβ peptides from senile plaques in the light of these possible factors will be necessary to assess the actual amount of nonstandard Aβ molecules, including $Aβ_{N3(pE)}$.

Our current goal in AD research is to specify the processes directly involved in AD pathogenesis. Although we have not yet reached the point of defining the actual rate-limiting metabolic steps regulating senile plaque formation, the present study provides new insights into future strategies. Because variations in both termini of Aβ seem deeply implicated regarding their ability to form plaques, we need to pay close attention to those proteases that influence the terminal structure of Aβ in the brain. These can be categorized into two groups on an enzymological basis. The first consists of two or more endopeptidases that cleave APP's and derivatives potentially producing Aβ peptides with diversified termini. The other includes a number of amino- and carboxy-exopeptidases that directly modify the Aβ peptides. If $Aβ_{N3(pE)}$ deposition proves to play a specific pathological role, the activity and localization of pyroglutamate-specific aminopeptidase(s) (McDonald and Barrett, 1986) may emerge as a candidate target for AD research. More specifically, we hypothesize that a reduced pyroglutamate-specific aminopeptidase activity may possibly contribute to early senile plaque formation. Lastly, the homophilic tendency of Aβ aggregation (Figure 9) implies that aggregation proceeds in a crystallization-like manner; local heterogeneity of Aβ metabolism could thus be an anti-AD factor working against plaque formation.

Experimental Procedures

Materials

Brain tissues used for the present study were from 28 aged individuals (aged 68–99; average, 84.7 ± 10.0) who displayed a wide variety of senile plaque formation in terms of density. Because only 3 of them had been diagnosed with AD based on clinical and pathological criteria (McKhann et al., 1984; Khachaturian, 1985), we did not make any comparison between AD and non-AD cases in the present study. For

Down's syndrome specimens, frontal cortices (Brodmann's area 8/9) were obtained at autopsy from 14 patients aged 31–64 years.

Synthetic peptides were produced using a peptide synthesizer ACT350 or ACT396 (Advanced Chemtech) as described (Saido et al., 1994a). The anti-$A\beta_{1-28}$ (Kanemaru et al., 1990) and anti-$A\beta_{1-5}$ (Saido et al., 1994a) antibodies have been described previously. An antiserum raised against $A\beta_{1-40}$ (Antiserum 1282) was a generous gift from Dr. Dennis J. Selkoe (Haass et al., 1992). The antibody specific to $A\beta_{N3(pE)}$ was produced using a synthetic peptide, pEFRHDC ($A\beta_{3(pE)-7}$ + C), conjugated to keyhole limpet hemocyanin and affinity-purified as described (Saido et al., 1992, 1994a). Other anti-Aβ antibodies, anti-$A\beta_{36-40}$ and anti-$A\beta_{38-42}$, were similarly prepared using synthetic peptides, CVGGVV and CGVVIA, respectively.

Immunohistochemistry

Brains obtained from the aged individuals and Down's syndrome patients were fixed in 10% buffered formalin for 1–4 weeks. Tissue blocks were cut, dehydrated, and embedded in paraffin, and serial sections were cut at 6 μm thickness. Sections were pretreated with 99% formic acid for 5 min before immunostaining (Kitamoto et al., 1987). Consecutive sections were immunostained with anti-Aβ antibodies by the avidin–biotin method using 3,3'-diaminobenzidine as chromogen and lightly counterstained with hematoxylin. The affinity-purified antibodies, anti-$A\beta_{1-5}$ and anti-Aβ 3(pE)-7, were used at a concentration of 2.5 μg/ml, while the anti-$A\beta_{1-28}$ antiserum was used at 400-fold dilution.

Biochemical Procedures

SDS–polyacrylamide gel electrophoresis (SDS–PAGE), Western blotting, and dot blotting were performed as previously described (Saido et al., 1992). Poly(vinylidene difluoride) membranes were used to immobilize blotted peptides. The affinity-purified antibodies were used at a concentration of 1 μg/ml, and the antisera were used at 1000-fold dilution. Alkaline phosphatase–conjugated anti–rabbit IgG antibody (Tago) and alkaline phosphatase substrate kit (Vector Red; Vector) were employed for visualization of immunoreactivity.

For biochemical quantitation of Aβ peptides in brains, we employed the following methods. Frozen cortices (1 g wet weight) were homogenized in a 10-fold volume of a buffer containing 10% SDS, 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 1 mM leupeptin, 1 mM diisopropyl fluoride, and 2.5 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 100,000 × g for 40 min at 20°C. The pellets were collected and rehomogenized in 1 ml of 99.0% formic acid for 60 min and centrifuged at 100,000 × g for 20 min at 20°C. The Aβ-containing supernatants were vacuum-dried and solubilized in 500 μl of SDS–PAGE solubilization buffer containing 8 M urea and 5% β-mercaptoethanol, followed by heat treatment at 100°C for 3 min. Aliquots (5 μl) were subjected to Western blotting using a 5%–20% gradient gel as previously described (Saido et al., 1994a), except that a chemiluminescence method using peroxdase-conjugated anti–rabbit IgG antibody (ECL kit, Amersham) was employed to visualize the immunoreactivity. The amount of the corresponding Aβ species was quantitated by calibrating the blot densities against known amounts of synthetic Aβ peptides (Shoji et al., 1992). Three separate experiments were performed to confirm the results.

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