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Alzheimer β/A4 amyloid precursor protein in human brain: Aging-associated increases in holoprotein and in a proteolytic fragment

(proteolysis/dementia)

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ABSTRACT **Alzheimer β/A4 amyloid precursor protein (APP) has been suggested to play a central role in the pathogenesis of Alzheimer disease. We have measured the content of different species of APP holoprotein and carboxyl-terminal fragments in human brains from young individuals, nondemented aged individuals, and aged individuals with Alzheimer disease. By using an antibody directed against the cytoplasmic domain of APP, five species were resolved. Three of these, of molecular masses 106, 113, and 133 kDa, represent presumptive immature and mature isoforms of APP holoprotein. Two smaller proteins, of molecular masses 15 and 19 kDa, represent presumptive proteolytic carboxyl-terminal fragments of APP. The 133-, 113-, 106-, and 15-kDa species were found in both grey and white matter, whereas the 19-kDa species was found only in grey matter. Total APP immunoreactivity (sum of all five species) and the levels of the 113-, 106-, and 15-kDa species were not significantly different in brain samples from young individuals, nondemented aged individuals, and aged individuals with Alzheimer disease. In contrast, the levels of the 133- and 19-kDa species increased 2- to 3-fold with age. A correlation was observed between the levels of the 133- and 19-kDa species, suggesting a possible precursor–product relationship. The size of the 19-kDa fragment indicated that it might have an intact β/A4 domain and therefore be amyloidogenic. The age-dependent increase either in a mature APP isoform and/or in a putative amyloidogenic fragment could explain why Alzheimer disease is associated with advanced age.**

Alzheimer disease (AD) is a common progressive encephalopathy of late life (1, 2). In most cases AD is sporadic, but familial forms also exist. Typically, the first manifestation of the disease is a subtle loss of short-term memory. Over the ensuing 6–20 years, cognitive function is progressively lost. The end stage of AD is characterized by almost total decortication. At this stage, the patient easily succumbs to secondary diseases such as infections (2).

The main neuropathological hallmarks of AD are neurofibrillary tangles, amyloid deposits, and neuronal cell death (2). Neurofibrillary tangles develop inside neurons of the brain and appear to be composed mainly of the microtubule-associated protein tau (3). Amyloid deposits, on the other hand, develop outside cells and are found both in the brain parenchyma and around meningeal and cerebral blood vessels. The amyloid deposit is relatively homogeneous being composed primarily of an ≈40-amino acid peptide (4, 5). This peptide, called the "β/A4 peptide," is generated by proteolytic cleavage of the Alzheimer β/A4 amyloid precursor protein (APP), an integral transmembrane protein (6–8). There are at least three different protein isoforms of APP with 695, 751, and 770 amino acid residues (6–11) generated by alternative splicing of mRNA derived from a single gene located on the long arm of chromosome 21 (6). When analyzed by SDS/polyacrylamide gel electrophoresis, APP isoforms appear as a group of proteins having apparent molecular masses between 93 and 149 kDa (12–16). APP undergoes posttranslational modifications (maturation) involving N- and O-linked glycosylation, tyrosyl sulfation, and phosphorylation (12–17). Maturation of APP decreases its mobility in SDS/polyacrylamide gels, leading to a higher apparent molecular mass (12, 14).

There is evidence that APP and the β/A4 peptide play a central role in the pathogenesis of AD. It has been reported that, in cell culture, synthetic β/A4 peptide can be toxic to mature neurons (18). Genetic studies have shown that familial AD can be associated with a point mutation in the coding sequence of the *APP* gene near the β/A4 domain (19). Further support for an association of AD with chromosome 21 and the *APP* gene is provided by the finding that individuals with trisomy 21 (Down syndrome) invariably develop typical AD pathology in the third or fourth decade of life (20).

In cultured cells, APP is rapidly turned over (14–16), and the rate of turnover can be increased by agents that either activate protein kinase C or inhibit protein phosphatases 1 and 2A (16). In the normal pathway of processing, APP is cleaved within the β/A4 region and the extracellular domain is released (21–23). This cleavage precludes amyloidogenesis and leaves the transmembrane and intracellular domains of the protein to be degraded by the cell. The proteolytic events that generate the β/A4 peptide from APP are unknown. It has been suggested, however, that in hereditary cerebral hemorrhage with amyloidosis (Dutch type), mutations close to the normal APP cleavage site may alter its binding to the processing enzyme (24). Mutations in the coding region of the *APP* gene could thereby alter processing of APP, leading to production of amyloidogenic fragment(s).

It has been reported that neither aging nor AD is associated with obvious changes in the levels of total APP mRNA or total APP protein in brain (25–27). Therefore, we undertook a study to determine whether aging or AD might be associated with alterations in the amounts of individual isoforms of APP or in their proteolytic fragments, or both. If such alterations were observed, they could provide insight into the age-associated generation of amyloid.

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Abbreviations: AD, Alzheimer disease; APP, Alzheimer β/A4 amyloid precursor protein; SDS, sodium dodecyl sulfate; AU, arbitrary unit(s).

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MATERIALS AND METHODS

The brains used in this study were either from young individuals (average age, 4.9 years; range, newborn to 18 years; $n = 19$), aged individuals with no known neurodegenerative disease (average age, 75 years; range, 62–86 years; $n = 11$), or individuals with AD (average age, 82 years; range 74–89 years; $n = 10$). The average postmortem interval for the young individuals was 15.6 hr (range, 3–26 hr), for the aged individuals with no known neurodegenerative disease was 26.4 hr (range, 3–46 hr), and for the AD patients was 26.2 hr (range, 3–46 hr). These three groups will be referred to as: young individuals, aged controls, and AD patients, respectively. The aged controls and AD patients, when analyzed as a group, will be referred to as combined aged individuals. Histopathological criteria used for the diagnosis of AD followed those outlined by the National Institutes of Health/American Association of Retired Persons Working Group (28), and only brains with a histopathological score more than 4 were considered to have AD (29). The rabbit antiserum against the cytoplasmic domain of human APP used in the present study has been described in detail elsewhere (16).

The brains were removed at autopsy, frozen, and stored at −70°C. The cerebral cortical grey matter and white matter from the frontal pole were carefully separated by dissection. The tissue was solubilized in 1% SDS by sonication for 10 s at maximal output (Microson ultrasonic cell disruptor, Farmingdale, NY). Sonication was followed by boiling for 5 min. Protein content was determined with the bicinchoninic acid method (30) (Pierce) with bovine serum albumin as standard. If samples were not analyzed immediately, they were stored at −70°C. After thawing, the samples were sonicated again. The samples were diluted with SDS sample buffer (31) to a final protein concentration of 0.60 mg/ml and separated on SDS/7.5–15% gradient polyacrylamide gels (10 μg or 30 μg per lane). The separated proteins were then electroblotted to nitrocellulose membranes (32), and the membranes were probed with affinity-purified rabbit antibody against the human APP-(645–694) fragment corresponding to the carboxyl-terminal region of the 695-amino acid APP isoform (≈3.0 μg/ml). After washing in Tris-buffered saline, pH 7.5/0.05% Tween-20, the nitrocellulose membranes were incubated with horseradish peroxidase-coupled goat anti-rabbit antibody (Amersham). After extensive washing, the immunoreactive proteins were visualized by autoradiography with a chemiluminescence detection system (ECL, Amersham). The autoradiograms were scanned with a soft-laser densitometer (Biomed Instruments, Fullerton, CA). The density of each band was compared with a standard curve that was obtained with various quantities of brain protein extract (3–100 μg per lane). For each APP species, one arbitrary unit (AU) corresponds to the content in 1 μg of the reference brain protein extract.

Comparisons between different age groups were made with the nonparametrical Wilcoxson's rank-sum test, where the groups were compared by relative ranks. Correlations between the amounts of the different APP fragments and intact APP holoproteins were determined as described (33).

RESULTS

Composition of APP Immunoreactivity in Human Brain. The APP immunoreactivity in grey matter was composed of five protein bands (Fig. 1) of 133, 113, 106, 19, and 15 kDa and designated APP 133, APP 113, APP 106, APP 19, and APP 15, respectively. In some individuals, APP 113 appeared as two closely spaced bands that could not be resolved by densitometry (data not shown). In white matter, protein species with molecular masses apparently identical to APP 133, APP 113, APP 106, and APP 15 but not to APP 19 could be detected

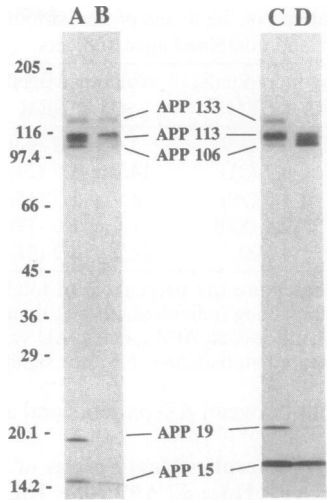

FIG. 1. (*Left*) APP immunoreactivity detected in samples of grey matter (lane A) and white matter (lane B) obtained from the frontal cortex of a 62-year-old woman with no known neurodegenerative disease. (*Right*) APP immunoreactivity in a sample of grey matter from the frontal cortices from an 82-year-old woman with AD (lane C) and a newborn boy (lane D). Each lane contained 30 μg of total protein. The molecular mass markers were: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.2 kDa).

(Fig. 1). Samples of grey and white matter from 16 brains from young individuals, aged controls, and AD patients were analyzed for APP 19. All 16 samples of grey matter contained APP 19, whereas none of the 16 samples of white matter contained APP 19. White matter contained all APP species other than APP 19 in essentially the same proportions as did grey matter. When antibody was preabsorbed with the synthetic APP-(645–694) fragment of the 695-amino acid isoform of APP, none of the five proteins was visualized.

Level of Total APP Immunoreactivity in Human Brain. The total APP immunoreactivity (i.e., the sum of the values for all five bands) in samples of grey matter from human frontal cortex is shown in Fig. 2. The amount of total APP immunoreactivity in samples from combined aged individuals was not statistically different from that in samples from young individuals ($P > 0.1$). Moreover, there was no difference in

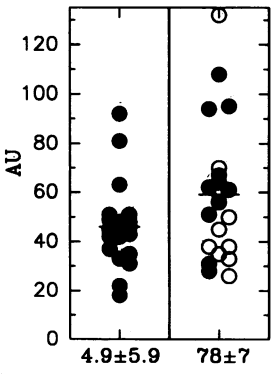

FIG. 2. Levels of total APP immunoreactivity (i.e., sum of all five species) in young individuals, aged controls, and AD patients. (*Left*) Young individuals (●). (*Right*) Aged controls (●) and AD patients (○). The mean values are indicated by bars. Mean ages ± SD for the two groups are shown beneath the figure. There was no difference ($P > 0.1$) between the levels of total APP immunoreactivity when comparing young individuals with combined aged individuals or when comparing aged controls with AD patients.

Table 1. Comparison of the levels of the various APP species in young individuals and combined aged subjects

Species	Young individuals, AU ± SEM	Combined aged, AU ± SEM	Significance
APP 133	1.7 ± 0.2 (3.7)	3.5 ± 0.3 (5.9)	$P < 0.001$
APP 113	9.7 ± 0.7 (21)	14.0 ± 1.7 (24)	NS
APP 106	9.4 ± 1.0 (20)	8.9 ± 1.7 (15)	NS
APP 19	3.7 ± 0.4 (8.0)	11.0 ± 1.0 (19)	$P < 0.001$
APP 15	22.0 ± 4 (48)	22.0 ± 3.9 (37)	NS

Data in parentheses are the percentage of total APP immunoreactivity (in AU) for young individuals ($n = 19$) and combined aged individuals ($n = 21$). For each APP species, AU values from the two groups were compared statistically. NS, not significant.

total APP content between AD patients and aged controls ($P > 0.1$).

Levels of High Molecular Mass Species of APP in Human Brain (APP 133, APP 113, and APP 106). The high molecular mass species of APP probably correspond to noncleaved APP holoprotein isoforms. The brains from the combined aged individuals contained on average 2.1 times more APP 133 than did brains from young individuals ($P < 0.001$; Fig. 3*A*). When the level of APP 133 was plotted against the age of the individual from which the sample was taken, the correlation coefficient obtained was 0.605 ($P < 0.01$). There was not a significant difference in the level of APP 133 between the brains from AD patients and those from aged controls. In the young individuals and in the combined aged individuals, APP 133 represented 3.7% and 5.9% of the total APP immunoreactivity, respectively (Table 1).

The level of APP 113 did not differ statistically in brain samples from the combined aged individuals compared with samples from young individuals ($P > 0.1$; Fig. 3*B*). Moreover, there was not a significant difference in the level of APP 113 between the brains from AD patients and those from aged controls. APP 113 represented 21% of the total APP immunoreactivity in the samples from young individuals and 24% of the total APP immunoreactivity in the samples from the combined aged individuals (Table 1).

The level of APP 106 did not differ statistically in brain samples from combined aged individuals compared with samples from young individuals ($P > 0.1$; Fig. 3*C*). Neither was there any difference in APP 106 levels between samples from AD patients and aged controls ($P > 0.1$). APP 106 represented 20% of the total APP immunoreactivity in the samples from young individuals and 15% of the total APP immunoreactivity in the samples from the combined aged individuals (Table 1).

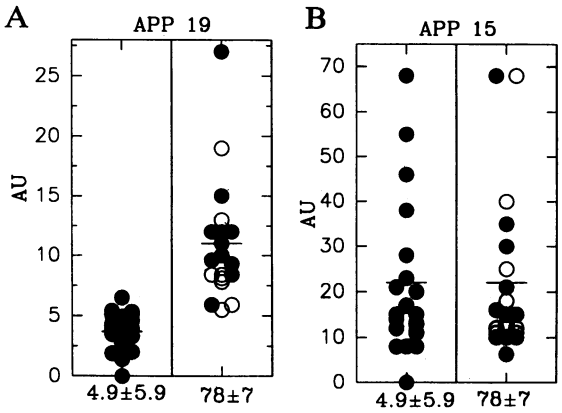

FIG. 4. The levels of carboxyl-terminal APP fragments in samples of grey matter from the frontal cortex from young individuals, aged controls, and AD patients. (*A*) APP 19. (*B*) APP 15. Mean values are indicated by bars. (*Left* in *A* and *B*) Young individuals (●). (*Right* in *A* and *B*) Aged controls (●) and AD patients (○). Mean ages ± SD for the young individuals and combined aged individuals are indicated. Samples from combined aged individuals contained more APP 19 than did samples from the young individuals ($P < 0.001$). There was no difference in the levels of APP 15 between the young individuals and combined aged individuals ($P > 0.1$).

The levels of the high molecular weight APP species did not correlate with the postmortem intervals (data not shown).

Levels of Low Molecular Mass Species of APP in Human Brain (APP 19 and APP 15). The antibody used in this study was directed against the carboxyl-terminal portion of APP. Therefore, the APP species of 15 and 19 kDa (Fig. 1) probably correspond to carboxyl-terminal fragments of APP containing the entire intracellular and transmembrane domains and part of the extracellular domain of APP.

Brain samples from the combined aged individuals contained 3.0 times more APP 19 than did samples from young controls ($P < 0.001$; Fig. 4*A*). The correlation coefficient obtained when levels of APP 19 were plotted against the ages of the individuals from which the samples were obtained was 0.684 ($P < 0.01$). No difference was seen between levels of APP 19 in samples from AD patients and aged controls ($P > 0.1$). APP 19 represented 8% of the total APP immunoreactivity in young individuals and 19% of the total APP immunoreactivity in the samples from the combined aged individuals (Table 1).

Levels of APP 15 were not different in brain samples from young individuals compared with the combined aged indi-

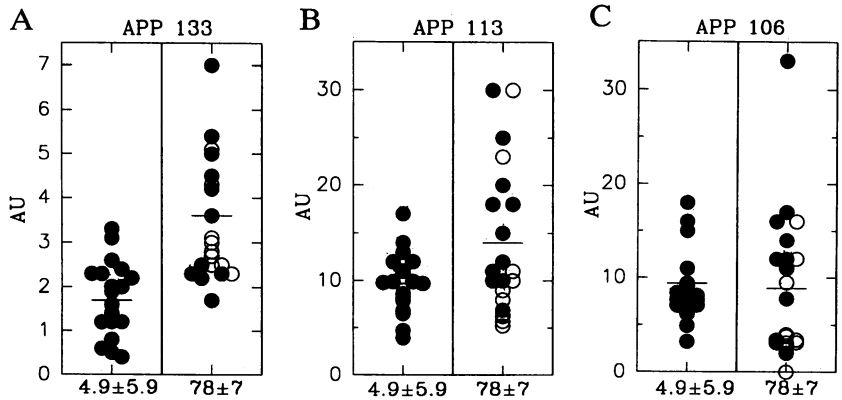

FIG. 3. Levels of full-length APP holoprotein species in samples of grey matter from the frontal cortex from young individuals, aged controls, and AD patients. (*A*) APP 133. (*B*) APP 113. (*C*) APP 106. Mean values are indicated by bars. (*Left* in *A*, *B*, and *C*) Young individuals (●). (*Right* in *A*, *B*, and *C*) Aged controls (●) and AD patients (○). Mean ages ± SD for the young individuals and combined aged individuals are indicated. Samples from combined aged individuals contained more APP 133 than samples from the young controls ($P < 0.001$). There was no difference in the levels of APP 113 and APP 106 between the young individuals and the combined aged individuals ($P > 0.1$).

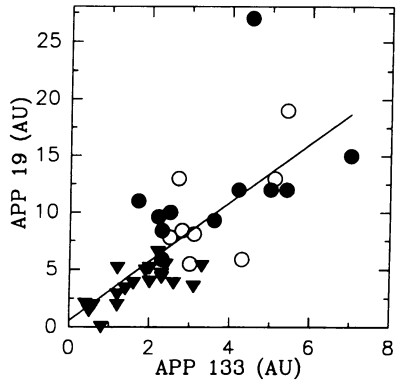

FIG. 5. Correlation between the levels of APP 133 and APP 19 in young individuals (▼), aged controls (●), and AD patients (○): $r = 0.735$, $P < 0.001$.

viduals ($P > 0.1$; Fig. 4*B*). Neither was there any difference between samples from AD patients and aged controls (Fig. 4*B*). APP 15 represented 48% of the total APP immunoreactivity in the samples from young individuals and 37% of the total APP immunoreactivity in the samples from the combined aged individuals (Table 1).

The levels of the low molecular mass APP species did not correlate with the postmortem intervals (data not shown).

Correlation Between the Levels of APP 133 and APP 19. The levels of APP 133 and APP 19 both increased with age. When the level of APP 19 was plotted against the level of APP 133 for each individual, a clear positive correlation ($r = 0.735$, $P < 0.01$) was obtained (Fig. 5). These results raise the possibility of a precursor–product relationship between the two immunoreactive species.

DISCUSSION

The major risk factor for AD is advanced age. Therefore, it is surprising that no consistent relationship, apart from amyloidosis, has been demonstrated between aging and levels of APP in brain (26). Brains from aged individuals and AD patients have been reported to contain the same amount of total APP protein as those of young individuals (27), a finding that was confirmed in the present study. The total APP immunoreactivity in human brain, measured by using an antibody against the carboxyl-terminal region of the 695-amino acid APP molecule in an immunoblotting assay, could be resolved into at least five different species of APP. Some of the APP species represented small fractions of the total APP immunoreactivity. Thus, aging- or AD-related differences in the levels of minor APP species might not significantly affect the total APP immunoreactivity in the samples. In the present study, the levels of two minor APP species, APP 133 and APP 19 (which on average corresponded to 4.9% and 13.5% of the total APP immunoreactivity of all brains studied), were shown to increase with age without significantly altering total APP immunoreactivity.

The molecular mass values for APP species with the three highest masses (APP 133, APP 113, and APP 106) indicate that they are full-length APP holoproteins (12–16). Based on information obtained with cell lines transfected with genes coding for different isoforms of APP (14, 15), we assign the following tentative identities to these proteins: APP 106 corresponds to immature 695-amino acid APP, APP 113 corresponds to a nonresolved mixture of mature 695-amino acid APP and immature 751- and 770-amino acid APP isoforms; and APP 133 corresponds to the mature 751- and 770-amino acid APP isoforms.

The brain samples also contained two APP species (APP 15 and APP 19) with considerably lower molecular mass values than the full-length APP holoproteins. APP 15 and APP 19 are

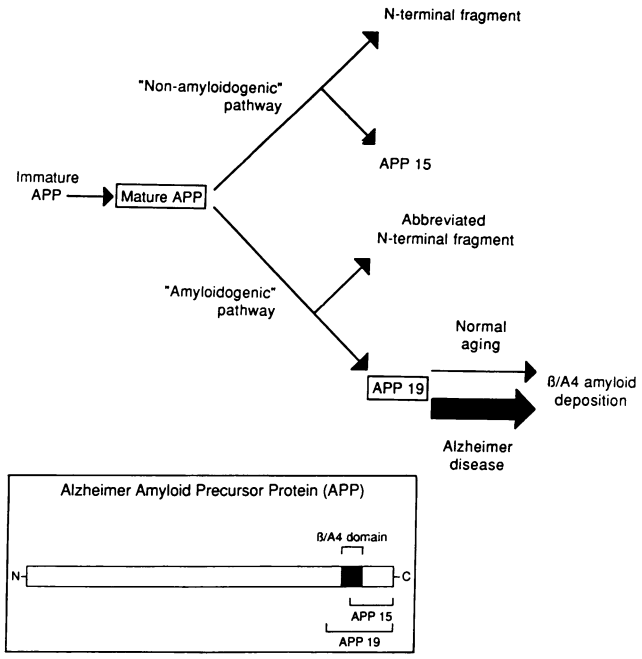

FIG. 6. Proposed mechanism for generation of β/A4 amyloid from APP in normal aging and AD. Aging increases the level of mature APP, saturating the "nonamyloidogenic" pathway that generates APP 15. The mature APP that cannot be degraded by the "nonamyloidogenic" pathway is instead degraded by an alternative, "amyloidogenic" pathway that generates APP 19. One difference between normal aging and AD is that APP 19 is converted more efficiently into β/A4 peptide in AD. (*Inset*) Proposed intra-β/A4 cleavage that generates nonamyloidogenic APP 15 from APP holoprotein and an alternative cleavage that generates amyloidogenic APP 19. The precise locus of the cleavage of APP holoprotein that leads to APP 19 is not known but is postulated to be at or slightly upstream from the amino terminus of the β/A4 domain. Boxes surround species whose levels increase with aging. For further discussion, see the text.

probably carboxyl-terminal proteolytic fragments of APP**. The molecular mass value of APP 15 is similar to that of the carboxyl-terminal APP fragment described by Esch *et al.* (23). That fragment was shown to be cleaved between amino acids Gln-15 and Leu-17 of the β/A4 region of APP. As a result of the cleavage in this region, β/A4 amyloid peptide could not be produced. The similarity of the molecular weight of APP 15 from human brain and that of the nonamyloidogenic fragment of Esch *et al.* (23) suggests that they may be cleaved at the same position. Therefore, APP 15 from the brain would represent the product of a nonamyloidogenic degradative pathway for APP.

Since APP 19 is 4 kDa larger than the presumably nonamyloidogenic APP 15, it is reasonable to hypothesize that APP 19 was generated by cleavage upstream of the β/A4 domain. With an intact β/A4 domain, APP 19 would be a putative amyloidogenic fragment. Whereas the level of APP 15 remained constant with aging, the level of APP 19 was 3-fold higher in samples from the combined aged individuals compared with samples from young individuals. Our data indicate that aging alters the metabolism of APP, leading to a substantial increase of APP 19. Consistent with the possible involvement of APP 19 in amyloidogenesis is the finding that APP 19 is found in grey, but

**The 19-kDa carboxyl-terminal fragment (APP 19) described in this paper does not comigrate in SDS/PAGE with the 19-kDa fragment observed in PC-12 cells (16). In the gradient gel system used in this paper, the 19-kDa fragment in PC-12 cells had an apparent molecular mass of 16 kDa. However, APP 15 (this paper) and the 15-kDa carboxyl-terminal fragment from PC-12 migrate with identical apparent molecular mass.

not white, matter of the brain. This distribution of APP 19 resembles the distribution of amyloid deposits seen in AD (2), whereas the probably nonamyloidogenic APP 15 is found in both grey and white matter.

The carboxyl-terminal amino acid residues of the three isoforms of APP are identical (6–11). Therefore, it is not possible at present to identify the isoform(s) from which APP 15 and APP 19 are derived. However, the level of APP 133, which probably represents the mature 751- and 770-amino acid APP isoforms, increased with age and was positively correlated with the levels of APP 19 in the brain samples studied. Thus, it is possible that aging leads to an increase of APP 133 that subsequently serves as a substrate for the generation of APP 19.

The five different species of APP shown in the present paper are also found in regions of the brain other than frontal cortex (data not shown). Moreover, various APP mRNAs and proteins have been observed in other nervous and nonnervous tissues of the body (6–11, 13). It remains to be determined whether the age-related changes described here can also be observed in other brain regions and in nonnervous tissues.

The levels of the APP species studied in the present paper were not different in samples from AD patients and aged controls. Therefore, it would appear that the increased levels of APP 133 and APP 19 seen in aging are not sufficient for generation of the amyloid deposits seen in AD. Our data suggest that some mechanism that produces β/A4 (e.g., from APP 19 or directly from APP 133) may work more effectively in AD than in normal aging to produce the heavily increased amounts of amyloid seen in AD. One hypothetical scheme by which amyloid might be produced in normal aging and AD is presented in Fig. 6.

Our general conclusions from these findings are that aging does not significantly affect the level of total APP immunoreactivity in human brain, but that it alters the relative distribution of different species of APP and APP carboxyl-terminal fragments. These results raise the possibility that, by increasing the levels of a putative amyloidogenic fragment, aging sets the stage for amyloid production. In other words, it is postulated that the formation of APP 19 or a similar fragment is necessary but not sufficient for the amyloidogenesis in AD. Since the levels of the putative amyloidogenic fragment were not higher in brains from AD patients than in brains from aged controls, a greater efficiency in the conversion of APP 19 to β/A4 amyloid peptide might be responsible for the accelerated formation of amyloid in AD.

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