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ORIGINAL PAPER

Intraneuronal Aβ immunoreactivity is not a predictor of brain amyloidosis-β or neurofibrillary degeneration

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Abstract Amyloid β (Aβ) immunoreactivity in neurons was examined in brains of 32 control subjects, 31 people with Down syndrome, and 36 patients with sporadic Alzheimer's disease to determine if intraneuronal Aβ immunoreactivity is an early manifestation of Alzheimer-type pathology leading to fibrillar plaque formation and/or neurofibrillary degeneration. The appearance of Aβ immunoreactivity in neurons in infants and stable neuron-type specific Aβ immunoreactivity in a majority of brain structures during late childhood, adulthood, and normal aging does not support this hypothesis. The absence or detection of only traces of reaction with antibodies against 4–13 aa and 8–17 aa of Aβ in neurons indicated that intraneuronal Aβ was mainly a product of α- and γ-secretases ($A\beta_{17-40/42}$). The presence of N-terminally truncated $A\beta_{17-40}$ and $A\beta_{17-42}$ in the control brains was confirmed by Western blotting and the identity of $A\beta_{17-40}$ was confirmed by mass spectrometry. The prevalence of products of α- and γ -secretases in neurons and β- and γ-secretases in plaques argues against major contribution of Aβ-immunopositive material detected in neuronal soma to amyloid deposit in plaques. The strongest intraneuronal $A\beta_{17-42}$ immunoreactivity was observed in structures with low susceptibility to fibrillar Aβ deposition, neurofibrillary degeneration, and neuronal loss compared to areas more vulnerable to Alzheimer-type pathology. These observations indicate that the intraneuronal Aβ immunoreactivity detected in this study is not a predictor of brain amyloidosis or neurofibrillary degeneration. The constant level of Aβ

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immunoreactivity in structures free from neuronal pathology during essentially the entire life span suggests that intraneuronal amino-terminally truncated Aβ represents a product of normal neuronal metabolism.

Keywords Intraneuronal amyloid-β · Plaques · Tangles · Down syndrome · Alzheimer's disease

Introduction

Neurofibrillary degeneration and brain amyloidosis with deposition of fibrillar amyloid β (Aβ) in plaques are diagnostic features of Alzheimer's disease (AD). Intracellular processing of amyloid precursor protein (APP) with β- and γ-secretases generates $A\beta_{1-40}$ and $A\beta_{1-42}$ in the endoplasmic reticulum, trans-Golgi network, and endosomal–lysosomal system [10, 19, 25, 62, 66]. An amino-terminally truncated 3-kd peptide ($A\beta_{17-40/42}$) is the product of APP cleavage with α- and γ-secretase. Cells may produce and secrete several species of Aβ, including $A\beta_{1-40}$, $A\beta_{1-42/3}$ and $A\beta_{17-40/42}$ [8, 23, 24].

Human neurons are Aβ-immunoreactive [11, 17, 22, 38, 39, 57]. The nature, distribution, and role of intraneuronal Aβ are the subject of controversy (see review by Takahashi [58]). Intraneuronal Aβ immunoreactivity has been localized in lipofuscin deposits [4, 65], cathepsin D-positive vesicles of lysosomal origin [11], multivesicular bodies within presynaptic and postsynaptic compartments [57], and intracellular and extracellular neurofibrillary tangles (NFTs) [1, 21, 26, 27, 34, 42]. Several studies of cytoplasmic Aβ immunoreactivity in neurons and glial cells in the human brain have been conducted to determine the properties of Aβ in human neurons and its role in fibrillar plaque formation [11, 17, 22, 39, 57]. The key observation has been the absence of [64] or only minimal intraneuronal Aβ immunoreactivity [22] in normal brain. Therefore, the appearance of or an increase in Aβ immunoreactivity has been suggested as a sign of neuronal pathology [22] leading to fibrillar plaque formation in the brain of people with AD [11, 15, 22, 39, 63].

Overexpression of APP, overproduction of Aβ, and early intracellular accumulation of Aβ [33] have been considered the foundation for early onset of AD pathology and functional deterioration in adults with Down syndrome (DS) in their 40's [22, 39, 63]. The loss of Aβ immunoreactivity in areas of plaque formation has led to the conclusion that neurons release intracellular Aβ, which initiates a seeding process leading to plaque formation [39]. According to Bahr et al. [2], the death of neurons, a prominent feature of AD, is associated with the release of oligomerized intracellular $A\beta_{42}$ into the surrounding milieu, which may stimulate the production of amyloidogenic fragments of APP, amplify the levels of intracellular Aβ in neighboring cells, and act as a nidus for the deposition of secreted Aβ.

The association of intracellular $A\beta_{42}$ with intraneuronal tangles has been considered an indication that the growing concentration of $A\beta_{42}$ may contribute to NFT formation [40]. The presence of altered aspartyl residues in intracellular NFTs has been interpreted as indicating that racemized Aβ peptides are involved in neurofibrillary degeneration [41, 52].

The pattern of intraneuronal Aβ immunoreactivity observed in the control cohort in our previous study [61] was in conflict with the hypothesis that Aβ immunoreactivity in neurons is the key and an early event in the cascade of pathology leading to AD. Therefore, the aim of this study was to reexamine the hypothesis that intraneuronal Aβ immunoreactivity is an early manifestation of Alzheimer-type pathology leading to the formation of fibrillar plaque and/or neurofibrillary degeneration. To test this hypothesis, we examined patterns of Aβ accumulation in the human brain during development, adulthood, and aging, and patterns of changes in the amount and distribution of Aβ in neurons in people with DS and DS/AD, and sporadic AD.

Materials and methods

Human tissue

The brains of 99 individuals were examined. Control brains included 32 cases, from 3 months to 102 years of age (3-, 6-, 11-, and 13-month-old infants; 4-, 8-, 14-year-old children; and adults from 23 to 102 years of age; 19 males and 13 females). DS brains included 31 cases from 3 weeks to 72 years old (infants that were 3 weeks, and 3, 6, and 9 months old and adults from 28 to 72 years of age; 15 males and 16 females). In the AD cohort, the brains of 36 subjects from 65 to 97 years of age (17 males and 19 females) were studied. Diagnostically, three subjects had a mild cognitive impairment (MCI) corresponding to global deterioration scale (GDS) stage 3 [45, 46], a frequent clinical precursor of AD. GDS stage 4 (mild AD) was reported for three subjects; stage 5 (moderate AD) for three subjects; stage 6 (moderately severe AD) for 11 subjects, and stage 7 (severe AD) for 16 subjects.

One brain hemisphere was fixed in 10% buffered formalin for 1.5 to several months and then dissected into 1 cm thick slabs. The tissue blocks were dehydrated for 5 days in 70% ethanol, then 2 days in 80% ethanol, and finally 1 week in 96% ethanol. The samples were infiltrated with polyethylene glycol (PEG) 400 (Merck #807,485) for 6 days (two changes of 3 days each, at room temperature) and with PEG 1000 for another 6 days (two changes of 3 days each, at 42°C). The slabs were embedded in fresh PEG 1000 [28]. The tissue blocks were cut serially at 50 μm thick sections. The immunoproperties of intracellular Aβ in tissues fixed in formalin for 1.5 to several months were also compared with immunostainings of sections from five brains (two DS and three AD) prefixed in 4% paraformaldehyde for 2 h, dissected into 1 cm thick slabs, fixed in 4% paraformaldehyde for the next 24 h, cryoprotected with 15 and 30% solutions of sucrose (4 days), frozen, and cut serially into 50 μm thick sections.

The methods selected for this study were approved by the New York State Institute for Basic Research (IBR) Institutional Review Board. The tissue samples were provided by the Silberstein Institute for Aging and Dementia at New York University, the Brain Bank at IBR, and the University of Miami Brain and Tissue Bank. In postmortem examination, all samples were identified only by an anonymous case number, and tissue was examined blind to clinical and demographic information.

Immunostaining

Several antibodies were applied to serial sections to evaluate Aβ immunoreactivity in cortical and subcortical structures, the cerebellum, and brainstem. Monoclonal antibodies (mAbs) 6E10 and 6F/3D were used for characterization of the amino-terminal portion of Aβ (Table 1). mAb 6E10 recognizes an epitope in residues 4–13 of Aβ (Signet Laboratories, 1:10,000) [30, 36, 59]. mAb 6F/3D recognizes an epitope in residues 8–17 of Aβ (Novocastra Laboratories Ltd; NCL-β-amyloid). The central portion of Aβ was detected with mAb 4G8, which recognizes an epitope in residues 17–24 of Aβ [29]. The carboxyl terminus of Aβ was characterized with pAbs purified from rabbit serum by epitope-specific affinity chromatography. These antibodies react with Aβ residues 32–40 (Catalog nr. 44–348) and 32–42 (Catalog nr. 44–344; BioSource International, Inc., CA, USA). The reactivity towards other species of Aβ peptides was eliminated through a series of preabsorption steps. Purified rabbit polyclonal antibodies, R164 and 165, specific for the carboxyl terminus of Aβ$_{1–42}$ (residues 35–42) were also used [37]. Fibrillar Aβ was detected with rabbit polyclonal antibody R262 produced by immunization of rabbits with fibrillar Aβ$_{1–42}$. Rabbit antibodies were purified according to the protocol described by Miller et al. [36].

Monoclonal antibody 6E10 reacts with Aβ both on western blots and in formalin fixed material [13, 14]. mAb 6E10 binds APP on western blots, as a native protein, but does not immunoreact with APP after fixation with formalin and dehydration. We have also shown that in cultures of cells overexpressing APP and formalin fixed and dehydrated both antibodies, 6E10 and 4G8 do not detect APP but they detect Aβ [13]. Also, the study of cultured cells with elevated levels of APP and C-terminal fragments of APP revealed that mAb 4G8 does not detect APP and C-terminal fragments of APP by immunocytochemistry [12] (Table 2).

The endogenous peroxidase in the sections was blocked with 0.2% hydrogen peroxide in methanol. To enhance immunoreactivity of Aβ, sections were treated with 90% formic acid for 30 min [31]. The sections were then treated with 10% fetal bovine serum in phosphate buffer solution (PBS) for 30 min to block nonspecific binding. The antibodies were diluted in 10% fetal bovine serum in PBS and were incubated with sections overnight at 4°C. The sections were washed and treated for 30 min with either biotinylated sheep anti-mouse IgG antibody or biotinylated donkey anti-rabbit IgG antibody diluted 1:200. The sections were treated with an extravidin peroxidase conjugate (1:200) for 1 h and the product of reaction was visualized with diaminobenzidine (0.5 mg/ml with 1.5% hydrogen peroxide in PBS). After immunostaining, the sections were lightly counterstained with cresyl violet.

Table 1 Mouse monoclonal and rabbit polyclonal antibodies used for immunocytochemistry

Type	Name	Epitope	Dilution	Source
mAb	6E10	4–13 aa Aβ	1:10,000	Signet Laboratories (developed at IBR)
mAb	6F/3D	8–17 aa Aβ	1:50	Novocastra
mAb	4G8	17–24 aa Aβ	1:8,000	IBR (Dr. R. Kascak)
pAb	44–348	32–40 aa Aβ	1:500	Biosource
pAb	44–344	32–42 aa Aβ	1:500	Biosource
pAb	R164	35–42 aa Aβ	1:500	IBR (Drs. D.L. Miller and P.D. Mehta)
pAb	R165	35–42 aa Aβ	1:500	IBR (Drs. D.L. Miller and P.D. Mehta)
pAb	R262	Fibrillar Aβ 1–42aa	1:200	IBR (Drs. D.L. Miller and P.D. Mehta)
mAb	Tau1	189–207 aa of tau	1:100,000	IBR (Dr. R. Kascsak)

Table 2 $A\beta_{n-40}$ and $A\beta_{n-42}$ concentration (pmol/g) in temporal cortex

Control case #	$A\beta_{n-40}$		$A\beta_{n-42}$	
	p3	p4	p3	p4
247	2	2	1	0.3
256	2	1	2	1
1,169	0.3	0.3	0.2[a]	0.2[a]

Brain samples were homogenized in formic acid as described in Materials and methods. Aliquots of the extracts were neutralized and subjected to PAGE and immunobloting. p4 bands migrated above the 3 kDa standards and bound mAb 6E10. p3 bands migrated near the 3 kDa standard and bound R162 or R226 but did not bind mAb 6E10

[a] There was only one band in this extract. Its level was too low to be revealed by mAb 6E10 and so could not be identified as p3 or p4

Phosphorylated tau protein of neurofibrillary tangles was detected with mAb Tau-1 (1:100,000). Tau-1 recognizes an epitope in residues 189–207 of the human tau sequence [16]. To obtain optimum staining with Tau-1, sections were treated with alkaline phosphatase (Sigma, Type VII-L, 400 μg/ml in PBS, pH 7.4, 0.01% H_2O_2) [20].

A three-point classification was used to estimate semi-quantitatively the difference of immunoreactivity with mAb 4G8 in four brain structures of 25 control adult subjects. The amygdala and cornu Ammonis were selected as structures susceptible to amyloidosis β and neurofibrillary degeneration. The lateral geniculate body and dentate nucleus were selected as structures resistant to AD pathology and almost free of plaques and NFTs even in severe AD. Grade 1 corresponded to weak immunoreactivity present in about 50% of neurons; grade 2 corresponded to moderate immunoreactivity in almost all neurons; and grade 3 corresponded to strong immunoreactivity present in almost all neurons in a given brain structure.

Sections from brain of DS subjects were used to determine whether intraneuronal Aβ immunoreactivity differs in people with an extra copy of the gene encoding APP. The difference between intraneuronal Aβ and amyloid in plaques was determined with antibodies detecting the amino- and carboxyl-terminus and the middle portion of Aβ peptide. The relationship of neurofibrillary changes to Aβ immunoreactivity in neurons was evaluated in brains of people with DS/AD and sporadic AD.

Partial purification and identification of Aβ peptides

Samples of the brain cortex (250 mg) from control males that were 31, 32 and 59 years-old were dispersed in 2.5 ml of 99% formic acid by brief sonification and centrifuged for 30 min at 100,000 g. Supernatants were dried by centrifugal evaporation, the residues were resuspended in 1 ml of 70% (v/v) formic acid and centrifuged as above. The solutions were subjected to size-fractionation on a 1 × 30 cm Pharmacia HR-12 column equilibrated with 70% formic acid as previously described [36]. The Aβ-containing fractions were identified by Western blotting and their contents were quantified by photodensitometry, as previously described [44]. The Aβ-containing fractions were dried under vacuum and were re-dried out of ammoniacal methanol to neutralize traces of formic acid. To solubilize Aβ peptides, each residue was treated with 250 μl of 50 mM ammonia and centrifuged as above, and the supernatant liquid was adjusted to pH 7.4 with KH_2PO_4.

Antibody R287, raised to $A\beta_{27-37}$ using the previously described methods [40] was purified on a peptide affinity column and was coupled to an Aminolink matrix (Pierce-Endogen) at a concentration of 360 pmol per 200 μl of settled matrix. The Aβ isoforms were immuno-adsorbed to 8 μl of R287-agarose during a 3 h incubation. Following washes with PBS and water the Aβ isoforms were eluted with 2 × 100 μl portions of 2.5% trifluoroacetic acid in 50% acetonitrile. About 50% of total Aβ peptides present in the cortex samples were recovered by this method, as evaluated by Western blotting. The shape and size of the Aβ band in Western blotting of the size-fractionated material and the immuno-purified peptide were the same, which suggested that the process did not selectively enrich any of the Aβ peptides.

Mass spectrometry and Western blotting

The peptide preparation isolated by immunoadsorption on R287-agarose was dissolved in 30 μl of 40% (v/v) formic acid and approximately 82% was loaded on a Symmetry® C18 nanoAcquity column (180 μm × 20 mm) as six sequential 4.1 μl injections. Following the sixth injection, the peptides were resolved on an Atlantis dC18 nanoAcquity column (100 μm × 100 mm) with a 20 min gradient of 10 to 50% acetonitrile in water containing 0.1% formic acid at flow rate of 0.4 μl/min. The eluate from the column was analyzed directly on a Qtof Micro (Waters Corp.) mass spectrometer equipped with a nanoflow electrospayer and scanned for *m/z* 50–1,500 at 1.1 s intervals. An external lock mass standard (leucine enkephalin; $m/z = 556.2771$) was analyzed at 11 s intervals through a separate orthogonal electrosprayer. The data were processed using MassLynx 4.0 (Waters Corp.) software, including Accurate Mass Measure and MaxEnt3 algorithms. Briefly, chromatograms were created from the data set by plotting a 1 Da window around the *m/z*'s of interest. The peaks were confirmed to have the component of interest by summing the mass spectra across the peak, determining the centroid m/z values, followed by deconvolution and transformation to the accurate mass value of the 1^+ ion. The resulting ion envelope encompassing mass isomers containing zero to four ^{13}C atoms was plotted to create the chromatogram of the specific peptide. For A$\beta_{17–40,}$ the 2^+ ion was by far the most prevalent and its ion envelope spanned m/z values from 1196.3 through 1198.7. For A$\beta_{1–40,}$ the 4^+ and 5^+ ions were the most prevalent, spanning m/z values from 1082.8 through 1084.8 and 866.4 through 868.0, respectively.

Western blotting was performed by a previously described method, which allows the detection of subfemtomol quantities of Aβ_{40} or Aβ_{42} [44]. Samples and appropriate standards were subjected to PAGE in tris-tricine 16% gels. Rabbit polyclonal antibodies R162 (raised to A$\beta_{31–40}$) and R226 (raised to A$\beta_{32–42}$) were used to detect the C-terminal sequences of Aβ isoforms. These antibodies are highly selective for their targets. R162 showed no cross-reactivity with a 100-fold excess of Aβ_{42} [44], and R226 is 2500-fold more reactive with Aβ_{42} than with Aβ_{40} [37]. Monoclonal antibody 6E10 [30] was used to detect the Aβ sequence 4–13 [59]. The blots were developed wth NBT and BCIP and the bands were quantified by photodensitometry [44].

Results

Age-associated changes of Aβ immunoreactivity in neurons in control and DS brains

Infants and children. In the majority of the brain structures examined, including the entorhinal cortex and neocortex, amygdala, and hippocampus, Aβ immunoreactivity was present in neurons of the 11 and 13 month-old normal infant brains and the 9 month-old infant diagnosed with DS (Fig. 1). At this age, Aβ-immunoreactivity was observed in only about 5–10% of the neurons. However, fine and randomly dispersed Aβ-immunoreactive granules were present in the cytoplasm in almost all neurons in the dentate gyrus. In infants, strong Aβ-immunoreactivity was found in clusters of cytoplasmic granules in almost all neurons in the magnocellular portion of the lateral geniculate body, but reaction in small neurons was rare and weak. In the brains of 4 and 8 year-old children, about half of neurons in the cortex and basal ganglia were Aβ-immunopositive, whereas in the brain of the 14 year-old child, the percentage of immunoreactive neurons was more than 50%.

Adults. In adults, Aβ immunoreactivity was present in the majority of the neurons, but the amount and pattern of distribution of immunopositive material showed a broad range of cell-type- and brain structure-specific differences. Strong Aβ immunoreactivity characterized all nuclei in the amygdala in adults, with the strongest reaction in the lateral and ventral subdivisions (mean grade, 2.3; SD ± 0.4). In the amygdala, numerous Aβ-positive cytoplasmic granules were concentrated at one pole of the cell and formed a perinuclear cap. In the cornu Ammonis (CA), Aβ immunoreactivity showed marked sector-specific differences. Strong and uniform reaction characterized neurons in the CA4 sector, and moderate reaction was observed in CA2 and 3 sectors, but Aβ immunoreactivity in neurons in the CA1 sector was much weaker and less uniform (mean grade in the CA, 1.8; SD ± 0.7). Apical dendrites in pyramidal neurons in the CA and subiculum proper were often marked with rows of Aβ-immunoreactive granules. Granule cells in the dentate gyrus contained numerous randomly dispersed Aβ-positive granules. Strong immunoreactivity appeared in large neurons, and moderate staining appeared in small neurons of the caudate nucleus and putamen. Reactions in neurons in the thalamus, globus pallidus, and n. accumbens were less uniform and weaker. Aβ immunoreactivity in cortical pyramidal neurons was stronger and more common than in cortical granule cells. In the cerebellum, a moderate amount of fine, granular Aβ- immunoreactive material was found in the majority of Purkinje cells, but in granule cells, the reaction was weak and was present only in a minority of cells. Very strong Aβ immunoreactivity was found in the cytoplasm in all neurons in the LGB and dentate nucleus (mean grade 2.8 ± 0.4), as well as in the nucleus olivaris.

Aged subjects. In general, in control people older than 65 years of age, Aβ immunoreactivity was reduced in about 20% of neurons of the amygdala, nucleus basalis of Meynert (NBM), cornu Ammonis, large neurons in the caudate-putamen, and cortex, as compared to control adults. However, the amount and pattern of Aβ immunoreactivity in the dentate gyrus, LGB, dentate nucleus, and nucleus olivaris inferior were comparable in aged subjects and normal adults.

In addition to the structure- and age-associated differences in neuronal Aβ immunoreactivity, there were interindividual differences between people of the same age and gender. Strong and uniform Aβ immunoreactivity was found in 44% of the control subjects; moderate and uniform in 40%, and weak and nonuniform in 16%. No difference between control and DS infants (Fig. 1) and adult with DS who has incipient neurofibrillary degeneration (28 years-old) was detected.

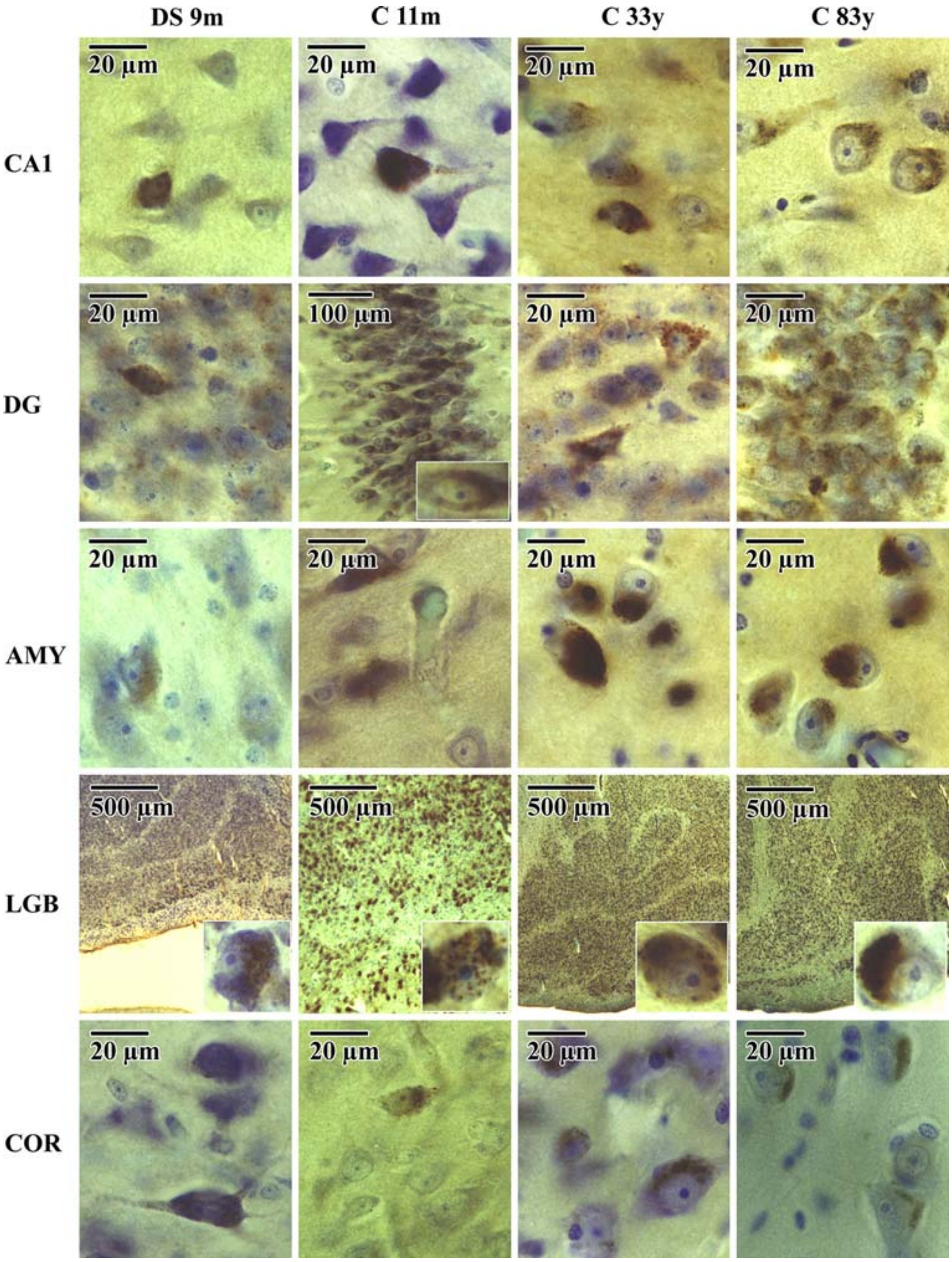

Fig. 1 Intraneuronal Aβ immunoreactivity with mAb 4G8 (17–24aa) in the CA1 sector, dentate gyrus (*DG*), amygdala (*AMY*), lateral geniculate body (*LGB*), and cortex (*COR*; temporal superior gyrus) in 9 month-old infant with DS, 11 month-old control infant, and 33 and 83 year-old control subjects (*C*) reveals age-associated and structure-specific differences. A few immunopositive neurons are present in CA1 sector, amygdala, and cortex in infants; however, immunopositivity is detectable in the majority of neurons in infant dentate gyrus and lateral geniculate body. In young adult (33 year-old) and aged subject (83 year-old) immunopositivity is present in the majority of neurons and is much stronger than in infants. Dense deposits are present in almost all neurons in the LGB and amygdala, and dispersed fine granular staining is present in the majority of neurons in the dentate gyrus. Loose granular immunopositive material appears in about 80% of neurons in CA1 sector and cortical neurons

Intraneuronal Aβ immunoreactivity in control brains with neurofibrillary degeneration and in DS/AD or sporadic AD

In the control group, mAb Tau-1 revealed neurofibrillary degeneration in the brains of all persons older than 43 years of age. In the entorhinal cortex, subiculum, and amygdala, the number of neurons with NFTs increases with age. The increase in Tau-1-positive material was paralleled by the reduction and loss of cytoplasmic Aβ immunoreactivity in affected neurons in the older subjects.

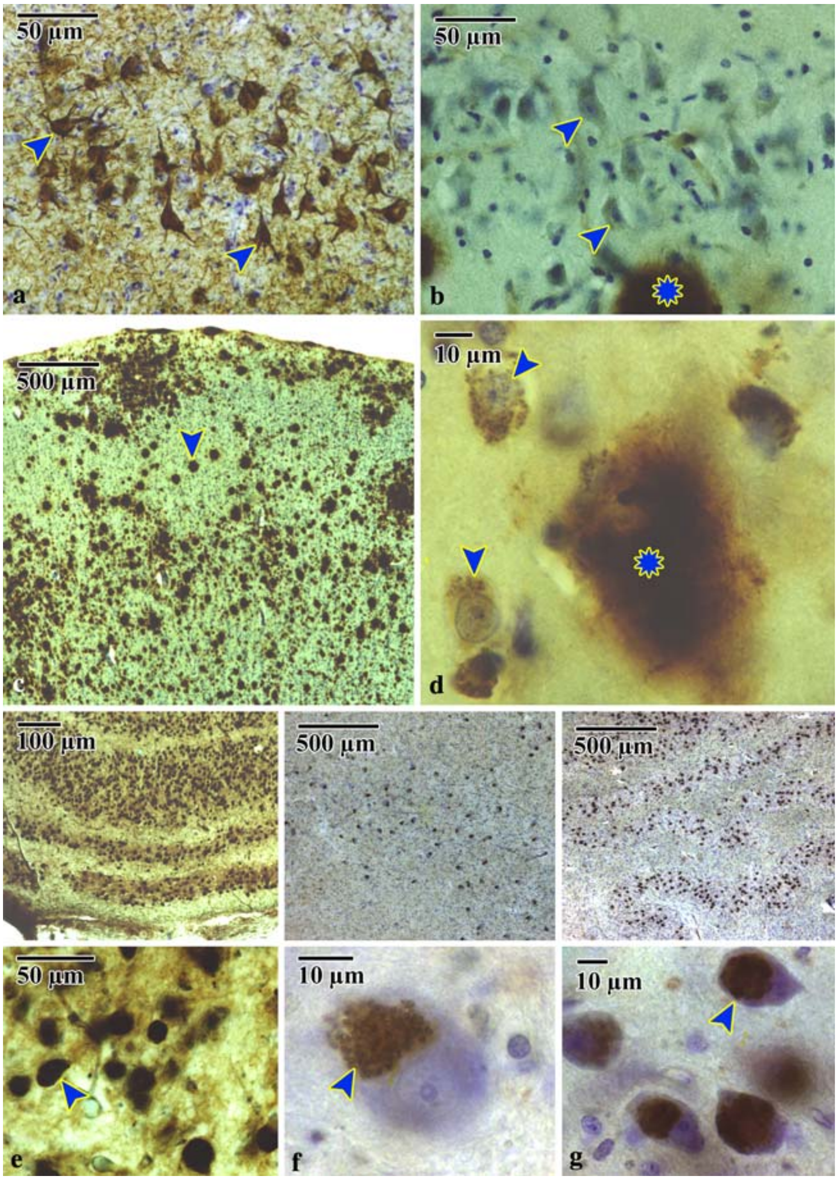

Fig. 2 Development of neurofibrillary tangles (*arrowheads* mAb Tau-1, **a**) in neurons in the second layer of the entorhinal cortex of a 43 year-old subject with DS/AD is associated with loss of cytoplasmic Aβ immunoreactivity (*arrowheads* mAb 4G8, **b**) without plaque formation in islands of stellate neurons. Amyloid plaque in the third layer (*asterisk*) is mAb 4G8-positive. The insula of a subject with mild AD (GDS 4) is affected by early and severe amyloidosis-β. Plaques are marked with *arrowheads* (**c**). mAb 4G8-positive material is present in both plaques (*asterisk*) and neurons (*arrowheads*, **d**). The strongest Aβ immunoreactivity is present in almost all neurons (*arrowheads*) in the lateral geniculate body (low and high magnification; AD, GDS 6; **e**), dentate nucleus (DS/AD, GDS 7; 72 year-old; **f**), and nucleus olivaris inferior (DS/AD, GDS 7; 72 years-old; **g**); however, amyloid plaques do not develop in these structures, even in severe AD

All subjects with DS older than 28 years-old were affected with neurofibrillary degeneration, and all people with DS older than 38 years of age had developed β amyloidosis. The increase in neurofibrillary degeneration, neuronal death, and β amyloidosis was associated with marked reductions of intraneuronal Aβ immunoreactivity in the entorhinal cortex (Fig. 2a, b), hippocampus, amygdala, NBM, and neocortex.

A similar pattern of reduction of Aβ immunoreactivity associated with neurofibrillary degeneration, amyloidosis β, and neuronal loss was found in people with MCI and subjects with sporadic AD (GDS stage 4–7). In severe AD (GDS stage 7), almost all neurons in the second layer of the entorhinal cortex were affected by neurofibrillary degeneration detected with mAb Tau-1. These neurons contained only traces of Aβ immunoreactivity or were free of cytoplasmic Aβ. However, in neocortex, the reduction of intracellular Aβ immunoreactivity was less pronounced (Fig. 2c, d).

Both in persons with DS/AD and persons with sporadic AD, the percentage of Aβ- immunopositive Purkinje cells was reduced by about 25% compared to age-matched control subjects. The majority of granule cells failed to manifest Aβ immunoreactivity. In the cerebellum, the decrease in neuronal Aβ immunoreactivity was observed in the almost total absence of intraneuronal NFTs.

In contrast to these patterns of reduction in Aβ immunoreactivity, a constant level of strong reaction was present in almost all neurons in LGB, dentate nucleus, and nucleus olivaris inferior in people with DS/AD or sporadic AD. These structures were almost free of NFTs and amyloid plaques in all AD subjects, including those with severe AD (Fig. 2e–g).

Immunoproperties of intraneuronal Aβ

The lack of Aβ immunoreactivity or presence of very few cytoplasmic grains immunoreactive with antibodies detecting an epitope in residues 4–13 (mAb 6E10) and 8–17 (mAb 6F/3D) and the presence of cytoplasmic immunoreactivity with antibodies detecting 17–24 (mAb 4G8), 32–42 (pAb 44–344), and 35–42 (pAbs R164 and 165), indicates that neurons harbor mainly amino-terminally truncated Aβ (Fig. 3a, b, c, d, e, f). Stronger staining of intracellular Aβ with pAb 44–344 (residues 32–42) and pAb R164 (35–42) than with pAb 44–348 (32–40), indicates that the major component of cytoplasmic Aβ in neurons is $A\beta_{17-42}$. Intraneuronal Aβ was not labeled with pAb R262 that detects fibrillar amyloid in cored plaques and in vessel wall (not shown). The pattern of immunostaining of Aβ in fibrillar plaques confirmed that parenchymal plaques contain mainly $A\beta_{1-42}$.

Aβ immunoreactivity in ghost tangles

While neuronal neurofibrillary changes were associated with loss of cytoplasmic Aβ immunoreactivity, some ghost tangles (extracellular NFTs) were Aβ-positive (Fig. 3g, h, i). In the sporadic AD group, among 31 examined subjects, Aβ-positive ghost tangles were found in 24 brains (77%). The proportion of subjects with Aβ-reactive ghost tangles increased from 50% in people with MCI to 70% in people with moderately severe AD and to 100% in people with severe AD. Aβ-positive ghost tangles were found in 100% of these subjects in the entorhinal cortex, in 87% in the CA1, 29% in the amygdala, 21% in the CA2 and CA4, 8% in the

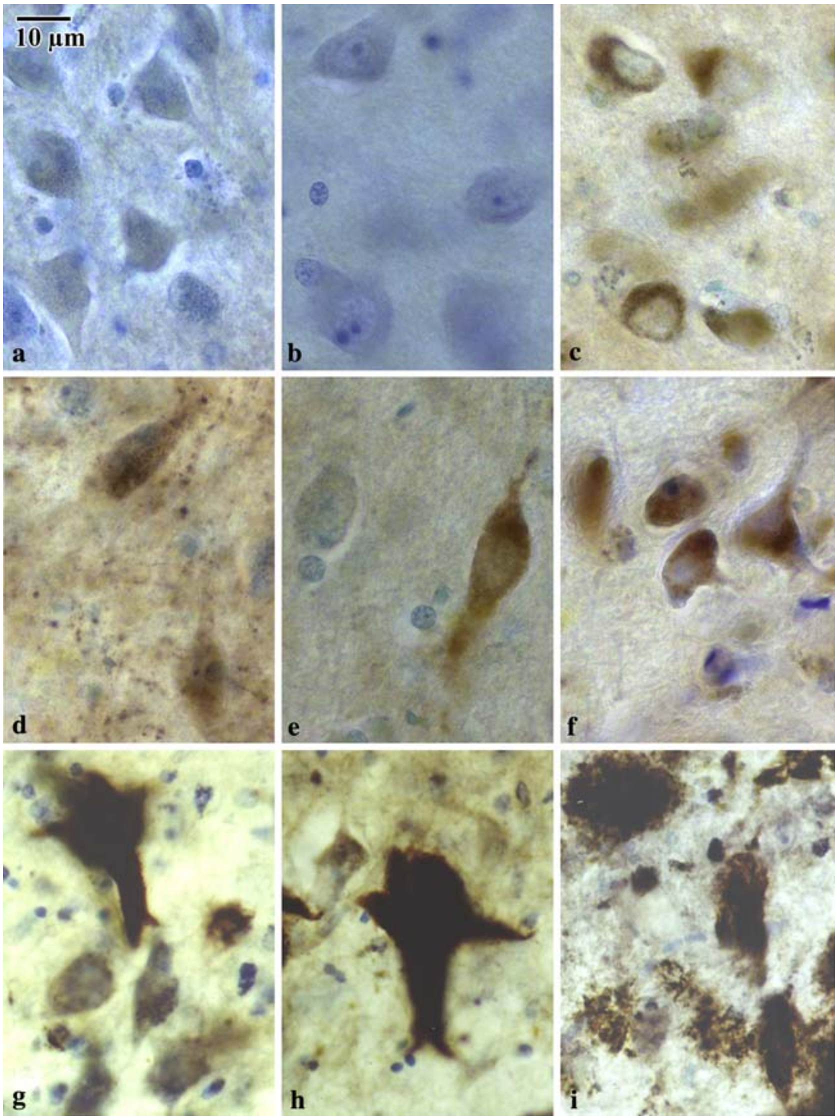

Fig. 3 **a–f** shows immunoreactivity of intraneuronal Aβ in the CA4 sector of a 32 year-old control subject. No reaction in neurons stained with mAb 6E10 (4–13 aa) (**a**) and mAb 6F/3D (8–17 aa) (**b**) indicates that in the majority of neurons, the amino-terminal portion of Aβ is not detectable. Immunoreactivity of intraneuronal Aβ is shown in sections stained with mAb 4G8 (17–24 aa) (**c**), pAb R164 (35–42 aa) (**d**), pAb 44–348 (32–40 aa) (**e**) and pAb 44–344 (32–42 aa) (**f**). In the brain of subjects with AD some extracellular ghost tangles contain full length Aβ peptides immunoreactive with antibodies 6E10 (**g**), 4G8 (**h**), and pAb 44–344 (**i**)

subiculum proper, and 4% in the CA3 and temporal cortex. Of AD subjects with Aβ-positive ghost tangles, they were numerous in 41%, moderate in number in 17%, and were rare in 21% of the subjects.

In 13 of 24 brains of persons with DS (54%), Aβ-immunoreactive ghost tangles were found in the entorhinal cortex, amygdala, and CA1 and CA2 sectors. The youngest subject with positive staining was 43 years of age at demise. Aβ-positive ghost tangles were found in the brains of all DS subjects from 55 to 72 years old. Of DS subjects in whom Aβ-immunoreactive ghost tangles were present, they were numerous in 46%, moderate in number in 39%, and rare in 15% of subjects.

In the control cases, no Aβ-immunoreactive ghost tangles were observed in spite of the presence of neurofibrillary changes in all subjects older than 43 years of age.

Aβ-positive ghost tangles were strongly immunoreactive with mAbs 6E10 (residues 4–13), 6F/3D (8–17), and 4G8 (17–24), and pAbs 44–344 (32–42) and R165 (35–42). The reaction with pAb 44–348 (32–40) was weak.

Aβ isoforms could be detected on Western blots of crude control brain extracts

Antibody R162 revealed 2 bands containing the $A\beta_{32-40}$ sequence (Fig. 4, lane 3), whereas antibody R226 revealed 2 bands containing the $A\beta_{33-42}$ C-terminal sequence (Fig. 4, lane 4). Immunoreactivity with mAb 6E10 (Fig. 4, lane 5) unequivocally showed that the upper band contained Aβ peptide sequences possessing amino acid residues 4–13. This antibody does not bind to $A\beta_{17-40}$ even at a 15-fold higher concentration (Fig. 4, lane 6). We found that the large amounts of other proteins in the extracts affected the electrophoretic migration rates and band shapes of the peptides, so that their migration rates did not unambiguously distinguish $A\beta_{1-40}$ from $A\beta_{17-40}$. Using synthetic Aβ standards we showed that substances in a crude brain extract retarded the migration of $A\beta_{17-40}$ so that it migrated at the rate of $A\beta_{1-40}$ (in the absence of brain extract).

Peptides with a defined C-terminus may have distinct N-termini, hence, we operationally define the p4 peptides as those that bind mAb 6E10 and migrate between the 3 and 6 kDa standards. Monoclonal antibody 6E10 binds to Aβ residues 4–13; therefore, p4 may contain peptides whose N-terminal residues are Asp_1–Arg_5. We define the p3 peptides as those that migrate near the 3 kDa standard and do not bind 6E10. The levels of Aβ40 peptides in three normal brain cortex samples were between 0.3 and 2 pmol/g of p3 and between 0.3 and 3 pmol/g, and the levels of Aβ42 peptides were between 0.2 and 2 pmol/g of p3 and between 0.2 and 1 pmol/g. Although these amounts are readily detectable, they are 3–4 orders of magnitude below the Aβ levels in an AD brain sample that we analyzed (not shown).

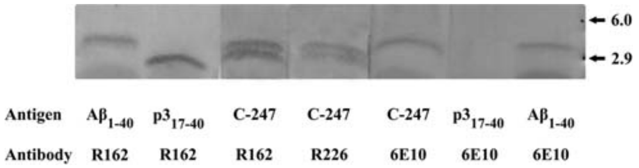

Fig. 4 Aβ isoforms in extracts from control brain 247 revealed by immunoblotting. A formic acid extract of cerebral cortex from brain C247 was prepared as described in the methods section. A portion of the dried extract was dissolved in PAGE sample buffer and a 15 μl aliquot containing 30 μg of protein (equivalent to 300 μg of tissue) was applied to the lanes developed with R162 and R226. Because mAb 6E10 is much less sensitive than R162 or R226, the formic acid extract was fractionated by size-exclusion as described in the methods section. The fraction containing peptides in the mass range 2,000–12,000 Da was concentrated, and an aliquot containing peptides from 12 mg of tissue was applied to the lane developed with 6E10. The extracts were subjected to PAGE and blotting along with synthetic Aβ standards and molecular mass markers as described in the text. The blots were developed with antibody R162 (to the C-terminus of $A\beta_{32-40}$), R226 (to the C-terminus of $Abeta_{33-42}$) or mAb 6E10 (to $A\beta_{4-13}$). *Lane 1* 2 fmol $A\beta_{1-40}$, *lane 2* 2 fmol $A\beta_{17-40}$, *lane 6* 150 fmol $A\beta_{17-40}$, *lane 7* 10 fmol $A\beta_{1-40}$. Molecular mass markers denoted on the right-hand margin are insulin single chains and aprotinin

Detection of $A\beta_{17-40}$ by mass spectrometry

We immunoprecipitated 650 fmol of Aβ peptides from brain C247 and characterized both the chromatographic behavior and the masses of the peptides by mass spectrometry using the coupled capillary HPLC-MS system. As shown in Fig. 5, the identity of the Aβ peptide $A\beta_{17-40}$ from the brain was confirmed by comparison to synthetic $A\beta_{17-40}$, which eluted at the same time and displayed the same m/z profile. Deconvolution and transformation of the $A\beta_{17-40}$ 2^+ ion spectrum to the 1^+ monoisotopic mass gave mass values of 2392.2852 Da (brain sample) and 2392.2813 Da (synthetic) compared to the theoretical value of 2392.2950 Da (4.1 ppm and 5.7 ppm errors, respectively). We also observed ion profiles consistent with $A\beta_{1-40}$ 4^+ and 5^+ ions in the mass spectra. The presence of detergent in the sample and the complexity of the chromatogram and spectra complicated the identification of other Aβ species.

Discussion

Aβ immunoreactivity in neurons in normal brain

Previous studies either did not find intracellular Aβ immunoreactivity in brains of control subjects [64] or

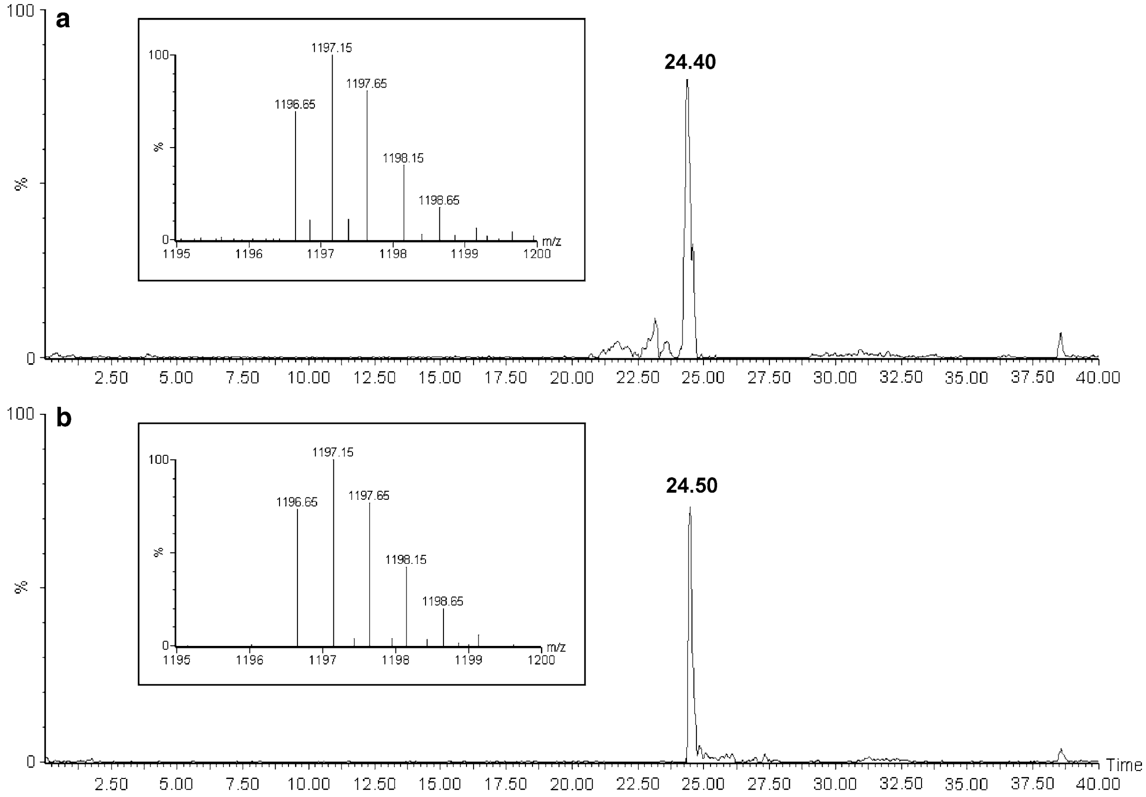

Fig. 5 Chromatographic elution profile of A$\beta_{17–40}$. Samples from (**a**) control brain C247 and (**b**) synthetic A$\beta_{17–40}$ were prepared and analyzed by LC-MS as described in "Materials and methods". The chromatograms show only the MS ion counts for the M+2H^{+} ions of A$\beta_{17–40}$ mass isomers containing zero to four ^{13}C atoms (*m/z* = 1196.3–1198.7). The ion intensities determined at 1.1 s intervals were summed, background subtracted and smoothed. The *y*-axis scale is 400 ions per second full scale (100%). The identification of A$\beta_{17–40}$ in the peaks at 24.4 (**a**) and 24.5 (**b**) minutes was demonstrated by the characteristic ion envelope obtained from the centroid accurate mass spectra for the A$\beta_{17–40}$ M+2H^{+} ions from each peak (24.2 to 24.7 min) shown in the *insets*

found only punctate Aβ immunoreactivity in a minority (15%) of examined adults [22]. Therefore, it was proposed that the accumulation of intraneuronal Aβ is involved in early AD pathology [17]. However, this study of control cases, from several months to 102 years of age demonstrates that intraneuronal Aβ immunoreactivity appears in the first year of life, increases in childhood, stabilizes in the second decade of life, and remains high throughout adulthood. Detection of stable intracellular Aβ immunoreactivity in neurons in control cases throughout the entire life may indicate that Aβ-immunoreactive material in the cell body reflects normal neuronal metabolism and is not neuronal pathology.

The observation that intraneuronal Aβ in control, DS/AD, and sporadic AD cases was almost exclusively amino-terminally truncated confirmed and extended the findings of the Mori et al. [39] study of people with DS and the Sergeant et al. [51] study showing that amino-truncated Aβ peptides represent more than 60% of all Aβ species in subjects with preclinical AD. Aβ peptides with N-terminal deletions exhibit enhanced peptide aggregation relative to full-length species [43] and retain the neurotoxicity and β-sheet structure. It was hypothesized that A$\beta_{17–42}$ peptides may initiate and/or accelerate plaque formation, perhaps by acting as nucleating centers that seed the subsequent deposition of relatively less amyloidogenic but apparently more abundant full-length Aβ [18, 43, 48]. Gouras et al. [17] considered intracellular Aβ_{42} accumulation to be an early event leading to neuronal dysfunction. However, the appearance of intraneuronal Aβ immunoreactivity in the first year of life, and the stable and strong immunoreactivity throughout adulthood, in the absence of morphological signs of cell injury or degeneration, suggests that Aβ detected in neurons with applied antibodies does not adversely affect cell structure. This lack of fibrillization and toxicity may indicate that these Aβ species remain in inert form. Possibly this inert state is maintained by binding with blockers of fibrillization and toxicity. The transport of Aβ within cytoplasmic vesicles or vacuoles [57] might be another factor preventing the expression of their cytotoxic activity. The detected intraneuronal

Aβ appears to be the physiological metabolite with unknown function. Higher levels of secreted APP and nonamyloidogenic secreted APP and lower levels of Aβ 40 in children with severely autistic behavior and aggression compared with controls [54] may indicate that modifications of APP processing and potentially Aβ trafficking are clinically significant in absence of neurodegeneration or neuronal loss.

The very strong Aβ immunoreactivity in the LGB, nucleus olivaris inferior, and dentate nucleus during the entire course of human adulthood and the absence of or minimal amyloid load in very severe AD oppose the hypothesis that strong intraneuronal Aβ immunoreactivity is a predictor of fibrillar plaque formation. Convergent findings in PS1 tg mice have been reported by Chui et al. [9].

This study suggests also that the intensity of intraneuronal Aβ immunoreactivity is not a predictor of neurofibrillary degeneration. The strongest Aβ immunoreactivity was observed in neurons in the LGB, nucleus olivaris inferior, and dentate nucleus, which remain free of neurofibrillary degeneration in very severe AD. The moderate Aβ immunoreactivity in the second layer of the entorhinal cortex and pyramidal neurons in the CA1, observed in this study, and the early onset of NFTs observed in these neuronal populations [5–7] also question the link between Aβ immunoreactivity and susceptibility to neurofibrillary degeneration.

The lack of pathological changes in brain structures with the strongest intraneuronal Aβ immunoreactivity during the entire human lifespan may indicate that amino-terminally truncated Aβ is a product of cell metabolism that does not (1) limit cell survival, (2) predispose to fibrillar Aβ deposition in plaques, nor (3) cause neurofibrillary degeneration.

Reduction of intraneuronal Aβ immunoreactivity in DS/AD and sporadic AD

In this study, the strongest intraneuronal Aβ immunoreactivity was found in control cases, compared to those with AD and in DS/AD. Significant weaker Aβ immunoreactivity in neurons in people with DS/AD and sporadic AD appear to be the result of AD pathology, including neurofibrillary degeneration and neuronal loss.

In the memory systems of persons with DS/AD or sporadic AD, neurofibrillary degeneration is the major cause of neuronal loss and, therefore, the major factor contributing to the reduction of intraneuronal Aβ. Over the course of 22 years, an AD patient loses 87% of the neurons in the CA1 and 63% in the CA4 sector, and 77% in the subiculum [5]. People with DS/AD older than 50 years of age are affected by severe loss of neurons including 98% loss in the second layer of the entorhinal cortex, 74% in the CA1 sector, 57% in the subiculum proper, and 71% in the amygdala [32, 60]. Large portions of surviving neurons in the brains of people with AD and DS/AD are affected with neurofibrillary changes, yet they show little or no Aβ immunoreactivity. These changes explain, in part, the loss of Aβ immunoreactivity in neurons in the plaque perimeter, as noted in persons with DS/AD [39]. The lower intraneuronal Aβ immunoreactivity observed in people with sporadic AD or DS/AD may also reflect a shift in APP processing. This shift would be from amino-terminally truncated Aβ accumulation in cell cytoplasm in normal brain to APP processing with enhanced generation and secretion of $A\beta_{1-40/42}$ in people with AD.

Accumulation of $A\beta_{1-40/42}$ in ghost tangles

Intracellular and extracellular NFTs have been shown to be Aβ immunoreactive [1, 21, 27, 34, 42, 49, 56]. However, studies of purified PHF revealed the absence of beta-pleated sheet conformation or any other characteristic of an amyloid [50]. It was proposed that the staining of intraneuronal PHF with antibodies to amyloid is due to the proximity of Aβ molecules to the PHF in the cytoplasm of neurons [47]. Deposition of Aβ within extracellular NFTs has been considered a secondary event [67].

Strong immunoreactivity of extracellular NFTs with antibodies 6E10 (residues 4–13), 6F/3D (8–17), 4G8 (17–24), 44–344 (32–42) and R165 (35–42) could be an indicator of the presence of full length of Aβ in extracellular space. The weak reaction of ghost tangles with pAb 44–348 (32–40) may suggest a lower concentration of this form of Aβ in the extracellular space. An increase in the percentage of Aβ-positive ghost tangles in people with progressing AD appears to reflect the increase in the percentage of neurons dying due to neurofibrillary degeneration and an increasing concentration of full length Aβ in the extracellular space. Spatial and temporal separation of plaques and Aβ-positive ghost tangles indicates that ghost tangles may bind Aβ, but do not initiate fibrillar plaque formation.

Interindividual differences in intraneuronal Aβ immunoreactivity

Intraneuronal Aβ immunoreactivity revealed some interindividual differences. Gouras et al. [17] observed increased apo-E immunoreactivity in $A\beta_{42}$-immunoreactive neurons and suggested that apo E might be a powerful modifier of intraneuronal Aβ accumulation. Neuron-generated apo-E may bind to neuronal Aβ and affect both its secretory pathway and its storage in the cell cytoplasm. The lack of apo-E in the offspring of βAPP tg mice crossed with apo-E knockouts reduces significantly the amyloid load [3]. Chronic inflammation is another factor that may influence amyloid formation [53].

In the human brain, there appear to be several pools of Aβ in equilibrium [35]. In this study, we demonstrate by the immunocytochemical method abundant intraneuronal amino-terminally truncated $A\beta_{17–40/42}$ with minor presence of the intraneuronal $A\beta_{1–40/42}$. The presence of the $A\beta_{17–40}$ peptide in control cortex samples was confirmed by mass spectrometry. However, in extracts from cerebral cortex we identified by Western blotting the $A\beta_{40}$ peptides that were amino-terminally truncated ($A\beta_{17–40}$) and full length $A\beta_{1–40}$ in similar quantities. We also detected both the $A\beta_{17–42}$ peptide and the full length $A\beta_{1–42}$ peptide, although the levels of the latter were 2 or 3 times lower than those of the amino-terminally truncated one. The differences between the Aβ peptides detected in the brain by the immunocytochemical and biochemical methods suggest that the Aβ peptides accumulated intraneuronally are mainly those amino-terminally truncated, while the full length peptides are mainly dispersed in extracellular space, and hence are not detected by the immunocytochemical method as intracellular deposits. Deposition of full length Aβ in ghost tangles and the increase of Aβ-positive ghost tangles could be considered an indicator of the presence of $A\beta_{1–42}$ in extracellular space and an increase of this extracellular form of Aβ in advanced stages of AD.

It appears that the immunocytochemical methods applied in this and other studies [11–14, 17, 22, 39] do not monitor the neuronal secretory pathway of APP processing leading to normal secretion of $A\beta_{1–40}$ and $A\beta_{1–42}$. Stern et al. [55] have shown that full length APP is extremely sensitive to fixation methods and its immunogenicity could be easily destroyed. The possibly enhanced secretion of these peptides in AD that may result in fibrillar Aβ deposition in plaques also is not detected. The lack of detection or poor detectability of the product of β- and γ-secretases in autopsy material might be the result of tissue preservation method, the minute amount of $A\beta_{1–40/42}$ that might be secreted without long-term storage, the prevalence of amino-terminally truncated Aβ masking the detection of $A\beta_{1–40/42}$, or the masking of the amino-terminal epitopes by interactions with other proteins. One may speculate that deposition of amino-terminally truncated Aβ might be up- or down-regulated by physiological and pathological factors independently of any changes in the secretory pathway.

This study suggests that neuron-type- and brain-structure-specific patterns of intraneuronal Aβ immunoreactivity, established in teenagers and maintained at a constant level during adulthood and aging, reflect normal cell metabolism rather than pathological changes. Reduction and loss of immunoreactivity in neurons with neurofibrillary changes appears to be a response to the progression of pathological changes in the neuron. The absence of a link between the age at onset and the progression of accumulation of intraneuronal Aβ-positive material in control subjects with the fibrillar plaque formation or neurofibrillary degeneration in people with AD indicates that the form of intraneuronal Aβ, detected by applied methods, does not predict plaque formation or neurofibrillary degeneration.

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References

1. Allsop D, Haga S, Bruton C, Ishii T, Roberts GW (1990) Neurofibrillary tangles in some cases of dementia pugilistica share antigens with amyloid beta protein of Alzheimer's disease. Am J Pathol 136:255–260
2. Bahr BA, Hoffman KB, Yang AJ, Hess US, Glabe CG, Lynch G (1998) Amyloid β-protein is internalized selectively by hippocampal field CA1 and neurons to accumulate amyloidogenic carboxyterminal fragments of the amyloid precursor protein. J Comp Neurol 397:139–147
3. Bales KR, Verina T, Dodel RC, Du Y, Altstiel L, Bender M, Hyslop P, Johnstone EM, Little SP, Cummins DJ, Piccardo P, Ghetti B, Paul SM (1997) Lack of apolipoprotein E dramatically reduces amyloid β-peptide deposition. Nat Genet 17:263–264
4. Bancher C, Grundke-Iqbal I, Kim KS, Wisniewski HM (1989) Immunoreactivity of neuronal lipofuscin, with monoclonal antibodies to the amyloid β-protein. Neurobiol Aging 10:125–132
5. Bobinski M, Wegiel J, Tarnawski M, Bobinski M, Reisberg B, de Leon MJ, Miller DC, Wisniewski HM (1997) Relationships between regional neuronal loss and neurofibrillary changes in the hippocampal formation and duration and severity of Alzheimer disease. J Neuropathol Exp Neurol 56:414–420
6. Braak H, Braak E (1991) Neuropathological staging of Alzheimer-related changes. Acta Neuropathol 82:239–269
7. Braak H, Braak E (1995) Staging of Alzheimer's disease-related neurofibrillary changes. Neurobiol Aging 16:271–284
8. Busciglio J, Gabuzda DH, Matsudaira P, Yankner BA (1993) Generation of β-amyloid in the secretory pathway in neuronal and non neuronal cells. Proc Natl Acad Sci USA 90:2092–2096

9. Chui DH, Tanahashi H, Ozawa K, Ikeda S, Checler F, Ueda O, Suzuki H, Araki W, Inoue H, Shirotani K, Takahashi K, Gallyas F, Tabira T (1999) Transgenic mice with Alzheimer presenilin 1 mutations show accelerated neurodegeneration without amyloid plaque formation. Nat Med 5:560–564
10. Cook DG, Forman MS, Sung JC, Leight S, Kolson DL, Iwatsubo T, Lee VM, Doms RW (1997) Alzheimer's Aβ (1–42) is generated in the endoplasmic reticulum/intermediate compartment of NT2N cells. Nat Med 3:1021–1023
11. D'Andrea MR, Nagele RG, Wang H-Y, Peterson PA, Lee DHS (2001) Evidence that neurons accumulating amyloid can undergo lysis to form amyloid plaques in Alzheimer's disease. Histopathology 38:120–134
12. Frackowiak J, Zoltowska A, Wisniewski H (1994) Non-fibrillar β-amyloid protein is associated with smooth muscle cells of vessel wlls in Alzheimer disease. J Neuropathol Exp Neurol 53:637–645
13. Frackowiak J, Miller DL, Potempska A, Sukontasup T, Mazur-Kolecka B (2003) Secretion and accumulation of Aβ by brain vascular smooth muscle cells from AβPP-Swedish transgenic mice. J Neuropathol Exp Neurol 62:685–696
14. Frackowiak J, Sukontasup T, Potempska A, Mazur-Kolecka B (2004) Lysosomal deposition of Aβ in cultures of brain vascular smooth muscle cells is enhanced by iron. Brain Res 1002:67–75
15. Glabe C (2001) Intracellular mechanisms of amyloid accumulation and pathogenesis in Alzheimer's disease. J Mol Neurosci 17:137–145
16. Goedert M, Spillantini M, Jakes R, Rutherford D, Crowther R (1989) Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. Neuron 3:519–526
17. Gouras GK, Tsai J, Naslund J, Vincent B, Edgar M, Checler F, Greenfield JP, Haroutunian V, Buxbaum JD, Xu H, Greengard P, Relkin NR (2000) Intraneuronal Aβ42 accumulation in human brain. Am J Pathol 156:15–20
18. Gowing E, Roher AE, Woods AS, Cotter RJ, Chaney M, Little SP, Ball MJ (1994) Chemical characterization of Aβ17–42 peptide, a component of diffuse amyloid deposits of Alzheimer disease. J Biol Chem 269:10987–10990
19. Greenfield JP, Tsai J, Gouras GK, Hai B, Thinakaran G, Checler F, Sisodia SS, Greengard P, Xu H (1999) Endoplasmic reticulum and trans-Golgi network generate distinct populations of Alzheimer β-amyloid peptides. Proc Natl Acad Sci USA 96:742–747
20. Grundke-Iqbal I, Iqbal K, Tung YC, Quinlan M, Wisniewski HM, Binder LI (1986) Abnormal phosphorylation of the microtubule associated protein τ (tau) in Alzheimer cytoskeletal pathology. Proc Natl Acad Sci USA 83:4913–4917
21. Guiroy DC, Mellini M, Miyazaki M, Hilbich C, Safar J, Garruto RM, Yanagihara R, Beyreuther K, Gajdusek DC (1993) Neurofibrillary tangles of Guamanian amyotrophic lateral sclerosis, parkinsonism dementia, and neuropathologically normal Guamanians contain a 4–4.5 kDa protein which is immunorective to anti-amyloid beta/A4 protein antibodies. Acta Neuropathol 86:265–274
22. Gyure KA, Durham R, Stewart WF, Smialek JE, Troncoso JC (2001) Intraneuronal Aβ-amyloid precedes development of amyloid plaques in Down syndrome. Arch Pathol Lab Med 125:489–492
23. Haass C, Schlossmacher MG, Hung AY, Vigo-Pelfrey C, Mellon A, Ostaszewski BL, Lieberburg I, Koo EH, Schenk D, Teplow DL, Selkoe DJ (1992) Amyloid β-peptide is produced by cultured cells during normal metabolism. Nature 359:322–325
24. Haass C, Hung AY, Schlossmacher MG, Teplow DB, Selkoe DJ (1993) β-amyloid peptide and 3 kDa fragment are derived by distinct cellular mechanisms. J Biol Chem 268:3021–3024
25. Hartmann T, Bieger SC, Bruhl B, Tienari PJ, Ida N, Allsop D, Roberts GW, Masters CL, Dotti CG, Unsicker K, Beyreuther K (1997) Distinct sites of intracellular production for Alzheimer's disease Aβ40/42 amyloid peptides. Nat Med 3:1016–1020
26. Hyman BT, Van Hoesen GW, Beyreuther K, Masters CL (1989) A4 amyloid protein immunoreactivity is present in Alzheimer's disease neurofibrillary tangles. Neurosci Lett 101:352–355
27. Ito H, Hirano A, Yen S-H, Katao S (1991) Demonstration of β amyloid protein containing neurofibrillary tangles in parkinsonism-dementia complex of Guam. Neuropathol Appl Neurobiol 17:365–373
28. Iqbal K, Braak H, Braak E, Grundke-Iqbal I (1993) Silver labeling of Alzheimer neurofibrillary changes and brain β-amyloid. J Histotech 16:335–342
29. Kim KS, Miller DL, Sapienza VJ, Chen CMJ, Bai C, Grundke-Iqbal I, Currie J, Wisniewski HM (1988) Neurosci Res Commun 2:121–130
30. Kim KS, Wen GY, Bancher C, Chen CMJ, Sapienza VJ, Hong H, Wisniewski HM (1990) Detection, quantitation of amyloid β-peptide with 2 monoclonal antibodies. Neurosci Res Comm 7:113–122
31. Kitamoto T, Ogomori K, Tateishi J, Prusiner S (1987) Methods in laboratory investigation. Formic acid pretreatment enhances immunostaining of cerebral and systemic amyloids. Lab Invest 57:230–236
32. Kuchna I, Wegiel J, Tarnawski M, Badmaev E, Kozlowski P, Pirttila T, Kivimaki T, Lehtimaki T, Visser F, Devenny D, Silverman W (2001) Alzheimer type pathology in the memory system of people with Down syndrome. J Neuropathol Exp Neurol 60:545
33. Lemere CA, Blusztajn JK, Yamaguchi H, Wisniewski T, Saido TC, Selkoe DJ (1996) Sequence of deposition of heterogenous amyloid β peptides and APOE in Down syndrome: implications for initial events in amyloid plaque formation. Neurobiol Dis 3:16–32
34. Masters CL, Multhaup G, Simms G, Pottgiesser J, Martins RN, Beyreuther K (1985) Neuronal origin of a cerebral amyloid: neurofibrillary tangles of Alzheimer's disease contain the same protein as the amyloid plaque cores and blood vessels. EMBO J 4:2757–2763
35. McLean CA, Cherny RA, Fraser FW, Fuller SJ, Smith MJ, Beyreuther K, Bush AI, Masters CL (1999) Soluble pool of Aβ amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. Ann Neurol 46:860–866
36. Miller DL, Currie JR, Mehta PD, Potempska A, Hwang Yu-Wen, Wegiel J (2003) Humoral immune response to fibrillar β-amyloid peptide. Biochemistry 42:11682–11692
37. Miller DL, Potempska A, Mehta PD (2007) Humoral immune responses to peptides derived from the β-amyloid peptide C-terminal sequence. Amyloid (in press)
38. Mochizuki A, Tamaoka A, Shimohata A, Komatsuzaki Y, Shoji S (2000) Aβ42-positive non-pyramidal neurons around amyloid plaques in Alzheimer's disease. Lancet 355:42
39. Mori C, Spooner ET, Wisniewski KE, Wisniewski TM, Yamaguchi H, Saido TC, Tolan DR, Selkoe DJ, Lemere CA (2002) Intraneuronal Aβ42 accumulation in Down syndrome brain. Amyloid J Protein Fold Disord 9:88–102
40. Murphy GM, Forno LS, Higgins L, Scardina JM, Eng LF, Cordell B (1994) Development of monoclonal antibody specific for the COOH-terminal of Aβ-amyloid 1–42 and its

immunohistochemical reactivity in Alzheimer's disease and related disorders. Am J Pathol 144:1082–1088

41. Payan IL, Chou S-J, Fisher GH, Man EH, Emory C, Frey WH (1992) Altered aspartate in Alzheimer neurofibrillary tangles. Neurochem Res 17:187–191
42. Perry G, Cras P, Siedlak SL, Tabaton M, Kawai M (1992) β protein immunoreactivity is found in the majority of neurofibrillary tangles of Alzheimer's disease. Am J Pathol 140:283–290
43. Pike CJ, Overman MJ, Cotman CW (1995) Amino-terminal deletions enhance aggregation of β-amyloid peptides in vitro. J Biol Chem 270:23895–23898
44. Potempska A, Mack K, Mehta P, Kim KS, Miller DL (1999) Quantification of sub-femtomole amount of Alzheimer amyloid β peptides. Amyloid 6:14–21
45. Reisberg B, Ferris SH, DeLeon MJ, Crook T (1982) The global deterioration scale for the assessment of promary degenerative dementia. Am J Psychol 139:1136–1139
46. Reisberg B (1988) Functional assessment staging (FAST). Psychopharmacol Bull 24:653–659
47. Rosenblum WI (1999) The presence, origin, and significance of Aβ peptide in the cell bodies of neurons. J Neuropathol Exp Neurol 58:575–581
48. Saido TC, Iwatsubo T, Mann DMA, Shimada H, Ihara Y, Kawashima S (1995) Dominant and differential deposition of distinct β-amyloid peptide species, AβN3(pE), in senile plaques. Neuron 14:457–466
49. Schwab C, Akiyama H, McGeer EG, McGeer PL (1998) Extracellular neurofibrillary tangles are immunopositive for the 40 carboxy-terminal sequence of β-amyloid protein. J Neuropathol Exp Neurol 57:1131–1137
50. Schweers O, Schonbrunn-Hanebeck E, Marx A, Mandelkow E (1994) Structural studies of tau protein and Alzheimer paired helical filaments show no evidence for beta structure. J Biol Chem 269:24290–24297
51. Sergeant N, Bombois S, Ghestem A, Drobecq H, Kostanjevecki V, Missiaen C, Wattez A, David J-P, Vanmechelen E, Sergheraert C, Delacourte A (2003) Truncated beta-amyloid peptide species in pre-clinical Alzheimer's disease as new targets for the vaccination approach. J Neurochem 85:1581–1591
52. Shapira R, Austin GE, Mirra SS (1988) Neuritic plaque amyloid is highly racemized. J Neurochem 50:69–74
53. Sheng JG, Bora SH, Xu G, Borchelt DR, Price DL, Koliatsos VE (2003) Lipopolisaccharide -induced-neuroinflammation increases intracellular accumulation of amyloid precursor protein and amyloid β peptide in APPswe transgenic mice. Neurobiol Dis 14:133–145
54. Sokol DK, Chen D, Farlow MR, Dunn DW, Maloney B, Zimmer JA, Lahiri DK (2006) High levels of Alzheimer beta-amyloid precursor protein (APP) in children with severely autistic behavior and aggression. J Child Neurol 21:444–449
55. Stern RA, Otvos L Jr, Trojanowski JQ, Lee VM (1989) Monoclonal antibodies to a synthetic peptide homologous with the first 28 amino acids of Alzheimer's diease beta-protein recognize amyloid and diverse glial and neuronal cell types in the central nervous system. Am J Pathol 134:973–978
56. Tabaton M, Cammarata S, Mancardi G, Manetto V, Autilio-Gambetti L, Perry G, Gambetti P (1991) Ultrastructural localization of β-amyloid, tau and ubiquitin epitopes in extracellular neurofibrillary tangles. Proc Natl Acad Sci USA 88:2098–2102
57. Takahashi RH, Milner TA, Li F, Nam EN, Edgar MA, Yamaguchi H, Beal MF, Xu H, Greengard P, Gouras GK (2002) Intraneuronal Alzheimer Aβ42 accumulates in multivesicular bodies and is associated with synaptic pathology. Am J Pathol 161:1869–1879
58. Takahashi RH, Nam EE, Edgar M, Gouras GK (2002) Alzheimer β-amyloid peptides: normal and abnormal localization. Histol Histopathol 17:239–246
59. Wang R, Sweeney D, Gandy SE, Sisodia S (1996) The profile of soluble amyloid β protein in cultured media. J Biol Chem 271:31894–31902
60. Wegiel J, Wisniewski HM, Morys J, Tarnawski M, Kuchna I, Dziewiatkowski J, Pirttilä T, Krivimäki T, Lehtimäki T, Lach B (1999) Neuronal loss and amyloid-β removal in the amygdala of people with Down syndrome. Neurobiol Aging 20:259–269
61. Wegiel J, Kuchna I, Miller D, Mehta P, Wegiel J, Wisniewski T, Reisberg B, Silverman W (2002) Intraneuronal accumulation of N-terminally truncated amyloid beta. J Neuropathol Exp Neurol 61:483
62. Wild-Bode C, Yamazaki T, Capell A, Leimer U, Steiner H, Ihara Y, Haass C (1997) Intracellular generation and accumulation of amyloid β-peptide terminating at amino acid 42. J Biol Chem 272:16085–16088
63. Wilson CA, Doms RW, Lee VM-Y (1999) Intracellular APP processing and Aβ production in Alzheimer disease. J Neuropathol Exp Neurol 58:787–794
64. Wirths O, Multhaup G, Czech C, Blanchard V, Moussaoui S, Tremp G, Pradier L, Beyreuther K, Bayere TA (2001) Intraneuronal Aβ accumulation precedes plaque formation in β-amyloid precursor protein and presenilin-1 double-transgenic mice. Neurosc Lett 306:116–120
65. Wisniewski KE, Maslinska D, Kitaguchi T, Kim KS, Goebel HH, Haltia M (1990) Topographic heterogeneity of amyloid β-protein epitopes in brains with various forms of neuronal lipofuscinoses suggesting defective processing of amyloid precursor protein. Acta Neuropathol 80:26–34
66. Xu H, Sweeney D, Wang R, Thinakaran G, Lo ACY, Sisodia SS, Greengard P, Gandy S (1997) Generation of Alzheimer's β-amyloid protein in the trans-Golgi in the apparent absence of vesicle formation. Proc Natl Acad Sci USA 94:3748–3752
67. Yamaguchi H, Nakazato Y, Shoji M, Okamoto K, Ihara Y, Morimatsu M, Hirai S (1991) Secondary deposition of beta amyloid within extracellular neurofibrillary tangles in Alzheimer-type dementia. Am J Pathol 138:699–705