Journal of Alzheimer's Disease 20 (2010) 1015–1028
DOI 10.3233/JAD-2010-091681
IOS Press

Immunohistochemical Visualization of Amyloid-β Protein Precursor and Amyloid-β in Extra- and Intracellular Compartments in the Human Brain

Leena Aho[a], Maria Pikkarainen[a], Mikko Hiltunen[a], Ville Leinonen[b] and Irina Alafuzoff[a,c,*]
[a]*Department of Clinical Medicine, Unit of Neurology, University of Eastern Finland, Kuopio, Finland*
[b]*Department of Neurosurgery, Kuopio University Hospital, Kuopio, Finland*
[c]*Department of Genetics and Pathology, Rudbeck's Laboratory, Uppsala University, Uppsala, Sweden*

Accepted 20 January 2010

Abstract. Amyloid-β (Aβ) peptide, a cleavage product of the amyloid-β protein precursor (AβPP), has been reported to be detected in the intracellular compartment. Most studies reporting the presence of intracellular Aβ are based on the use of immunohistochemistry. In this study, the presence of AβPP and Aβ was assessed by applying immunohistochemistry in postmortem human brain tissue samples obtained from 10 neurologically intact subjects, the youngest being 2 years of age, one aged with mild cognitive impairment, 14 neurologically diseased, and in one brain biopsy sample obtained from a subject with normal pressure hydrocephalus. Intracellular immunoreactivity was detected in all ages independent of the disease state or existence of extracellular Aβ aggregates with all antibodies directed to AβPP, with three Aβ antibodies (4G8, 6E10, and 82E1), clones that are unable to distinguish Aβ from AβPP. These results suggest that it is AβPP rather than Aβ that is detected intracellularly when using the antibodies listed above. Furthermore, the staining results varied when different pretreatment strategies were applied. Interestingly intracellular Aβ was detected with antibodies directed to the C-terminus of Aβ (neoepitope) in subjects with Alzheimer's disease. The lack of intracellular immunoreactivity in unimpaired subjects, when using antibodies against neoepitopes, may be due to a lack or a low level of the protein that is thus undetectable at light microscopic level by immunohistochemistry method. The staining results and conclusions depended strongly on the chosen antibody and the pretreatment strategy and thus multiple antibodies must be used when assessing the intracellular accumulation of Aβ.

Keywords: Amyloid-β, immunohistochemistry, intracellular, postmortem brain

INTRODUCTION

Already by the late 1980s, intracellular amyloid-β peptide (iAβ) [1] was reported to be seen in the brains of both subjects with Alzheimer's disease (AD) and controls applying an antibody that is commonly used in neuropathological diagnostics (clone 4G8). A review of the literature reveals that while applying various commercial antibodies (Table 1), iAβ has been reported to be detected in cell culture, in the brains of wild and transgenic animals, in brains obtained from subjects with Down's syndrome, AD, and HIV, and in young drug abusers as well as in brains obtained from children and aged without any known neurological disorders [1–23]. It is noteworthy and disturbing that there is considerable variation in results not only while applying a different antibody but even with the

*Correspondence to: Irina Alafuzoff, Department of Genetics and Pathology, Rudbeck's Laboratory, Uppsala University, Dag Hammarsköldsväg 20, SE-75815, Uppsala, Sweden. E-mail: irina.alafuzoff@genpat.uu.se.

ISSN 1387-2877/10/$27.50

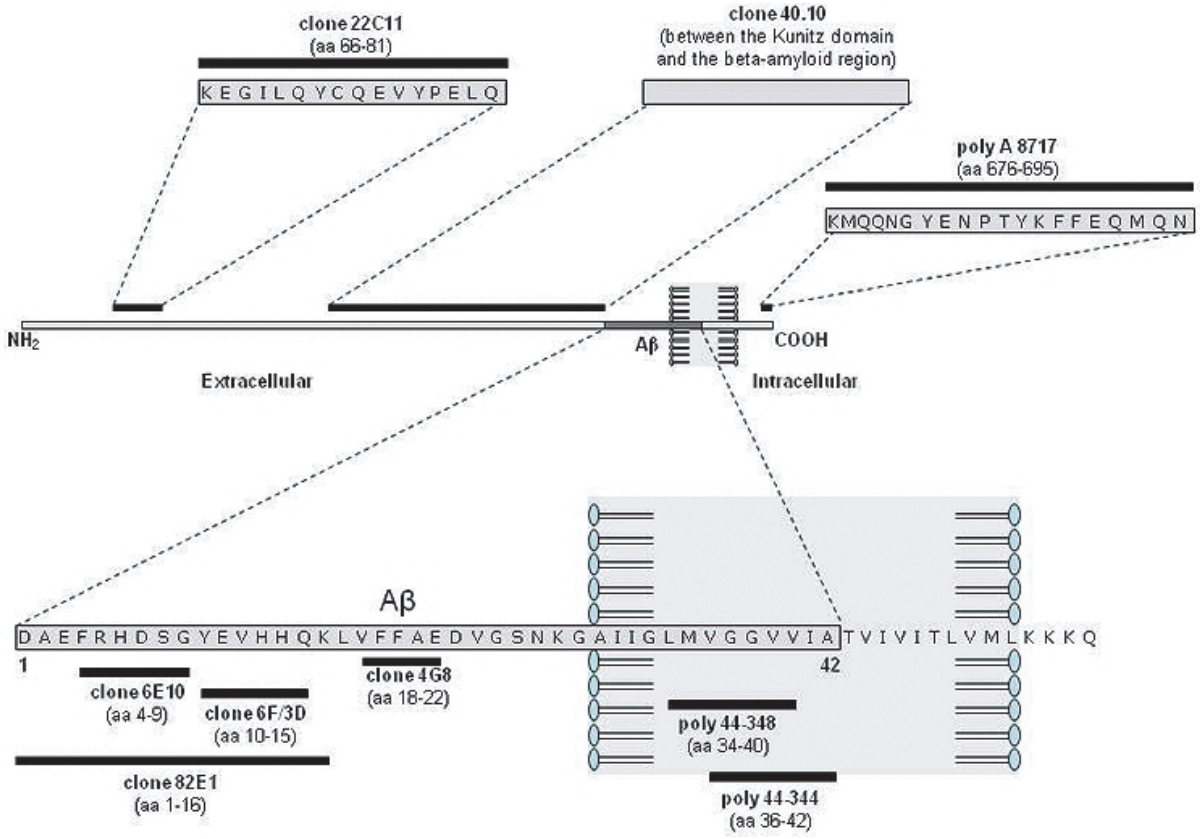

Fig. 1. A schematic presentation of the amyloid-β protein precursor (AβPP) where the epitope regions recognized by the antibodies used in this study are marked with black (AβPP/Aβ) and gray bars (Aβ neoepitopes). Clone 82E1 is raised against Aβ_{1-16} (Immuno-Biological Laboratories). Clone 6E10 is raised against Aβ_{1-17} (Signet) [3,56,57]. Clone 6F3D is raised against Aβ_{8-17} (DakoCytomation) [3,58]. Clone 4G8 (Signet) is raised against Aβ17–24 (Signet) [3,58]. Clone 12F4 is raised against Aβ_{1-42} (Covance) and is reactive to C-terminus of Aβ and is specific for the isoform ending at the 42nd amino acid. Clone 22C11, is raised against recombinant Alzheimer precursor A4 fusion protein; clone 40.10, is raised against the sequence between Kunitz protease inhibitor domain and the beta-amyloid region (Novocastra). The specificity of polyclonal antibodies as provided by the manufacturer: poly 44–348 (Biosource/Invitrogen), poly 44–344 (Biosource/Invitrogen), and poly A 8717 (Sigma). Aβ, amyloid-β.

same antibody (see Table 1).

Aβ consists of 40–43 amino acids and is a cleavage product of the transmembrane amyloid-β protein precursor (AβPP), encoded as a single-copy gene on chromosome 21 [24–28]. AβPP is widely expressed in the brain. The cleavage of AβPP has been reported to occur when AβPP is located at the plasma membrane, endoplasmic reticulum, endosomal and lysosomal membranes [29], trans-Golgi network [30], and mitochondrial membrane [31]. Thus, the cleavage product, Aβ, could be expected to be found in the intracellular compartment.

Most studies reporting the presence of iAβ (Table 1) are based on the use of immunohistochemistry (IHC). In IHC, antibodies, which recognize a specific sequence of amino acids, are used, i.e., an antibody recognizes usually only small part of a longer peptide (Fig. 1). With respect to both AβPP and Aβ, numerous commercial and in house monoclonal and polyclonal antibodies are available. As is seen in Fig. 1, some of the amino acid sequences, i.e., epitopes, are shared by Aβ and AβPP. It has been demonstrated that clone 6E10, which is specific for the sequence 4–13 of human Aβ, detects not only Aβ but also AβPP [32,33], and clone 4G8, which is directed to the mid-portion of Aβ, reacts to Aβ and AβPP derivatives in Western blots [34]. Thus, intracellular staining with monoclonal antibodies such as 6E10 or 4G8 may be detecting AβPP, various AβPP derivatives, or Aβ. Only in the 1990s did antibodies against the C-terminus (neopitopes) of Aβ (Aβ_{40} and Aβ_{42}), i.e., antibodies that are able to differentiate Aβ from AβPP, become available. It is noteworthy that despite the reports indicating that many of the commonly used antibodies are unable to distinguish Aβ from

Table 1
Published reports concerning intracellular labeling of Aβ in human diseases and animal models

Specificity of antibody	Materials		Intracellular IR of Aβ	Authors, year
	Study carried on	Disease / animal model		
$A\beta_{40}$ mAb or pAb	Human	Normal	+	Gouras et al. 2000 [11], D'Andrea et al. 2001 [7], Wegiel et al. 2007 [23]
			−	Gyure et al. 2001 [13], Nagele et al. 2002 [17], Cataldo et al. 2004 [4], Ohyagi et al.2007 [19]
		AD	+	Gouras et al. 2000 [11], D'Andrea et al. 2001 [7], Cataldo et al. 2004 [4], Wegiel et al. 2007 [23]
			−	Nagele et al. 2002 [17], Ohyagi et al. 2007 [19]
		Down syndrome	+	Gouras et al. 2000 [11], Gyure et al. 2001 [13], Cataldo et al. 2004 [4], Wegiel et al. 2007 [23] [54]
			−	Mori et al. 2002 [16]
	Animals	Wild	−	Kuo et al. 2001 [49], VanBroeck et al. 2008 [55]
		Transgenic	+	Lord et al. 2006 [33]
			−	Kuo et al. 2001 [49], VanBroeck et al. 2008 [55]
	Cell cultures		+	Gouras et al. 2000 [11]
$A\beta_{42}$ mAb or pAb	Human	Normal	+	Akiyama et al. 1999 [3], Gouras et al. 2000 [11], Gyure et al. 2001 [13], Nagele et al. 2002 [17], Cataldo et al. 2004 [4], Ohyagi et al. 2007 [19], D'Andrea et al. 2001; 2002; 2003 [7,9,39], Wegiel et al. 2007 [23]
			−	Mochizuki et al. 2000 [15], Gyure et al. 2001 [13]
		AD	+	Akiyama et al. 1999 [3], Gouras et al. 2000 [11], Mochizuki et al. 2000 [15], Nagele et al. 2002 [17], Wang et al. 2002 [22], Cataldo et al. 2004 [4], Ohyagi et al. 2007 [19], D'Andrea et al. 2001; 2002; 2003 [7–9,39], Wegiel et al. 2007 [23]
		Down syndrome	+	Gouras et al. 2000 [11], Gyure et al. 2001 [13], Mori et al. 2002 [16], Cataldo et al. 2004 [4], Wegiel et al. 2007 [23]
	Animals	Wild	+	VanBroeck et al. 2008 [55]
			−	Kuo et al. 2001 [49]
		Transgenic	+	Lord et al. 2006 [33], Oakley et al. 2006 [18], VanBroeck et al. 2008 [55]
			−	Kuo et al. 2001 [49]
	Cell cultures		+	Gouras et al. 2000 [11], Nagele et al. 2002 [17]
clone 4G8	Human	Normal	+	Grunke-Iqbal et al. 1989 [1], LaFerla et al. 1997 [14], Akiyama 1999 et al. [3], Gomez-Ramos et al. 2007 [10], Ohyagi et al. 2007 [19], Wegiel et al. 2007 [23]
		AD	+	Grunke-Iqbal et al. 1989 [1], LaFerla et al. 1997 [14], Akiyama et al. 1999 [3], Cataldo et al. 2004 [4], Gomez-Ramos et al. 2007 [10], Ohyagi et al. 2007 [19], Wegiel et al. 2007 [23]
		Down syndrome	+	Cataldo et al. 2004 [4], Wegiel et al. 2007 [23]
		HIV	+	Green et al. 2005 [12]
	Animals	Wild	+	Cruz et al. 2006 [6], VanBroeck et al. 2008 [55]
			−	Sheng et al. 2003 [21]
		Transgenic	+	Sheng et al. 2003 [21], Cruz et al. 2006 [6], Lord et al. 2006 [33], Oakley et al. 2006 [18], VanBroeck et al. 2008 [55]
clone 6E10	Human	Normal	−	Akiyama et al. 1999 [3], Wegiel et al. 2007 [23]
		AD	−	Akiyama et al. 1999[[3], Wegiel et al. 2007 [23]
		Down syndrome	−	Wegiel et al. 2007 [23]
		HIV	+	Green et al. 2005 [12]
	Animals	Wild	+	Cruz et al. 2006 [6], Kuo et al. 2001 [49]
			−	VanBroeck et al. 2008 [55]
		Transgenic	+	Cruz et al. 2006 [6], Kuo et al. 2001 [49], Lord et al. 2006 [33], Knobloch et al. 2007 [32]
			−	VanBroeck et al. 2008 [55]
clone 6F / 3D	Human	Normal	−	Akiyama et al. 1999 [3], Wegiel et al. 2007 [23]
		AD	−	Akiyama et al. 1999 [3], Wegiel et al. 2007 [23]
		Down syndrome	−	Wegiel et al. 2007 [23]

IR – immunoreactivity, mAb – monoclonal antibody, pAb – polyclonal antibody, + intracellular IR seen, − no intracellular IR noted.

AβPP, clone 4G8 was still being used as late as 2009 to detect iAβ in subjects with HIV [2,12].

In addition to the use of antibodies with variable specificities, the reports listed in Table 1 are carried out on the tissue obtained from either humans or animals. It has earlier been shown that species differences may to some extent influence the findings and thus the results obtained from animal studies are not as such directly comparable with those seen in humans [35, 36]. Therefore, some of the variability listed in Table 1 might be related to species differences. However, even when applying the same antibody or investigating the same species, the results have varied as is seen from Table 1. Another possible explanation for the discrepant results is varying antigen retrieval methods [19,37–40]. It has been demonstrated that a heating protocol can often enhance the staining whereas formic acid pretreatment alone is not sufficient to visualize intracellular immunoreactivity (IR) [19,39]. These reports have concluded that the existence of intracellular labeling may be underestimated, since many previous investigations employed only formic acid pretreatment. In summary, the tissue characteristics, choice of antibody, and modifications of pre-treatments strategy can significantly affect the outcome of an IHC labeling experiment.

The principal objective of this study was to test the reliability of the IHC technique while assessing iAβ in the routine diagnostic human postmortem material. The presence of AβPP and Aβ in the extra- and intracellular compartments was analyzed while applying the tissue microarray technique (TMA), several commercial antibodies, and several antigen retrieval methods.

MATERIAL AND METHODS

Case selection

This study was performed on surgical biopsy material and postmortem human brain tissue. The surgical sample was obtained from a patient who underwent intracranial pressure monitoring with frontal cortical biopsy for suspected normal pressure hydrocephalus. The postmortem brain tissues were sampled from the cases that had undergone an autopsy including a neuropathological examination. A total of 26 cases were included in this study; 1 surgical patient and 25 postmortem cases.

Surgical biopsy

A 75-year old female subject was operated due to normal pressure hydrocephalus. A right frontal 12 mm burr hole was made under local anesthesia and then cylindrical brain biopsies of 2 mm in diameter and 3 mm in length were taken through the burr hole. Half of the sample was fixed in 10% buffered formalin overnight and then embedded in paraffin. Seven-μm-thick serial sections were cut and placed on the SuperFrost® Plus-slides. The remaining half of the sample was placed in an Eppendorf tube and frozen at −70 °C.

Postmortem material

The demographics of investigated subjects are summarized in Table 2. There were 10 unimpaired subjects (age at death 2–92 years), a 100 year old female with mild cognitive impairment, 7 subjects with AD (age at death 57–87 years), and 7 subjects with frontotemporal lobar degeneration with TAR DNA binding protein 43 positive inclusions (FTLD-TDP-43) (age at death 50–93 years). The rapidity of death was classified as proposed by Hynd and colleagues in 2003 [41]. At autopsy, the brain was placed in 10% buffered formaldehyde for at least 1 week, and then cut into 1-cm-thick coronal slices. The brain specimens were taken from 16 standard regions and embedded in paraffin. For each case, the AD related Braak stage was assessed as described earlier [42]. Accumulation of eAβ was assessed in three neocortical sections in all cases while applying clone 4G8 and the results are given as Aβ aggregates seen or not seen. The selected neuroanatomical region in the aged was the hippocampal region, in AD the temporal cortex, and in FTLD-TDP-43 the frontal cortex. The HE stained slides of 7-μm thick sections were used to select regions for the core samples to be embedded into the TMA blocks. One of the TMA blocks contained samples from subjects who had been between the ages of 2 to 100 years at death, referred to as the TMA-aging (TMAa) the other TMA block contained samples from demented subjects, i.e., TMA disease (TMAd). From each case in the TMAa, four core samples were taken to ensure the sampling of possible eAβ. For the TMAd from each AD case, a sample from temporal cortex and from each FTLD-TDP-43 case, a sample from frontal cortex was taken. Each core in TMAa and TMAd block measured 2.0 mm in diameter as recommended by Kauppinen and colleagues. The tissue microarray (TMA) block was constructed as described earlier [43,44]. The core tissue sample from the donor blocks was taken using Beecher Instrument's Manual Tissue Arrayer 1 instrument and each core sample was inserted into previously made holes of the recipient block. The maximum

Table 2

Demographics, cases included in the tissue microarray blocks (TMA) of aging (TMAa) and disease (TMAd)

Case	Gender	Age at death, years	Pre-mortem neurological status/diagnosis	Primary cause of death / rapidity of death	Postmortem delay, hours	Fixation time, days	AD-related pathology		Core sample	
							HPτ Braak stage	eAβ in neocortex	taken from	found in
1	F	2	unimpaired	septic infection, slow	108	28	0	no	hippocampus with entorhinal cortex	TMAa
2	M	12	unimpaired	cerebral infarction, slow	96	72	0	no	hippocampus with entorhinal cortex	TMAa
3	M	22	unimpaired	cardiac infarction, slow	97	46	0	no	hippocampus with entorhinal cortex	TMAa
4	M	32	unimpaired	intoxication, slow	35	72	0	no	hippocampus with entorhinal cortex	TMAa
5	F	42	unimpaired	neoplasia, slow	24	45	0	no	hippocampus with entorhinal cortex	TMAa
6	M	52	unimpaired	cardiac insuffience, slow	88	46	1	yes	hippocampus with entorhinal cortex	TMAa
7	M	62	unimpaired	pulmonary embolus, fast	144	23	0	no	hippocampus with entorhinal cortex	TMAa
8	F	72	unimpaired	pulmonary embolus, fast	96	94	2	yes	hippocampus with entorhinal cortex	TMAa
9	M	82	unimpaired	cardiac infarction, fast	35	29	0	no	hippocampus with entorhinal cortex	TMAa
10	F	92	unimpaired	pulmonary embolus, fast	72	25	2	yes	hippocampus with entorhinal cortex	TMAa
11	F	100	mild cognitive impairment	cacexia, slow	4	na	2	yes	hippocampus with entorhinal cortex	TMAa
12	M	57	AD	cardiac insuffience, slow	8	88	6	yes	temporal cortex	TMAd
13	F	66	AD	pneumonia, slow	7	22	5	yes	temporal cortex	TMAd
14	F	73	AD	pneumonia, slow	3	79	5	yes	temporal cortex	TMAd
15	F	77	AD	cardiac infarction, fast	24	32	5	yes	temporal cortex	TMAd
16	F	79	AD	cardiac infarction, fast	5	75	6	yes	temporal cortex	TMAd
17	F	84	AD	pneumonia, slow	10	70	5	yes	temporal cortex	TMAd
18	F	87	AD	pneumonia, slow	14	56	4	yes	temporal cortex	TMAd
19	M	50	FTLD-TDP43	pneumonia, slow	22	38	0	no	frontal cortex	TMAd
20	F	57	FTLD-TDP43	pneumonia, slow	24	77	1	no	frontal cortex	TMAd
21	M	61	FTLD-TDP43	pneumonia, slow	7	46	0	no	frontal cortex	TMAd
22	F	68	FTLD-TDP43	pneumonia, slow	3	na	0	no	frontal cortex	TMAd
23	F	70	FTLD-TDP43	pneumonia, slow	38	57	0	no	frontal cortex	TMAd
24	F	73	FTLD-TDP43	pneumonia, slow	12	31	1	no	frontal cortex	TMAd
25	F	93	FTLD-TDP43	pneumonia, slow	5	10	1	no	frontal cortex	TMAd

AD- Alzheimer's disease, HPτ – hyperphosphorylated τ, eAβ- extracellular β-amyloid aggregates, na – not available, TMA- Tissue Microarray Technique, TMAa – TMA aging, TMAd – TMA disease, FTLD-TDP43- Frontotemporal lobar degeneration with TAR DNA binding protein 43 positive inclusions.

Table 3
Antibodies and dilutions

Name	Source / code	Clone	Immunogen	Dilution	Material/sections stained
Amyloid β (N)	Immuno-Biological Laboratories / 10323	82E1	Recognizes aa residues 1–16	1:100 1:500 1:1000[a]	TMAa, TMAa TMAa, TMAd, b
Amyloid β #	Signet / 9320	6E10	Recognizes aa residues 1–17 Epitope aa 4–9	1:1000 1:2000[a] 1:4000	TMAa TMAa, b, # TMAa
Amyloid β	Dakocytomation / M0872	6F/3D	Recognizes aa residues 8–17 Epitope aa 10–15	1:20 1:50[a] 1:100	TMAa TMAa, TMAd TMAa
Amyloid β #	Signet / 9220	4G8	Recognizes aa residues 17–24 Epitope aa 18–22	1:1000 1:2000[a] 1:4000	TMAa TMAa, TMAd, b, # TMAa
Amyloid β 40	Invitrogen / 44–348	rabbit	Reactive to aa residues 34–40	1:500 1:1000[a]	TMAa TMAa, TMAd, b
Amyloid β 42	Invitrogen / 44–344	rabbit	Reactive to aa residues 36–42	1:500 1:1000[a]	TMAa TMAa, TMAd, b
Amyloid β 42	Signet	12F4	Reactive to aa residues 36–42	1:1000	TMAa, TMAd
Amyloid precursor protein A4#	Chemicon / MAB348	22C11	Recognizes aa 66–81 of the N-terminus pre-A4 molecule	1:100[a]	TMAa, b, #
Amyloid precursor protein	Novocastra / NCL-APP	40.10	Recognizes extracellular portion of APP between the Kunitz protease inhibitor and the beta-amyloid region	1:40[a]	TMAa, b
Amyloid precursor protein C-terminal	Sigma / A8717	rabbit	Recognizes the C-terminus of APP	1:1000[a]	TMAa, b

mAb – monoclonal antibody, pAb – polyclonal antibody, aa – amino acid. [a]The optimal dilution to visualize intracytoplasmic labeling. b – Staining applied on biopsy. # staining applied on consecutive mirror section. TMAa – tissue microarray block of aging, TMAd – TMA of disease.

depth of the used punch was 8 mm, and the thickness of the recipient block was 10 mm. The resulting TMA blocks were warmed in an oven for 10 min at 57 °C to promote the adherence of the core sample to the paraffin of the recipient block. Finally, the TMA blocks were placed upside-down in stainless steel molds for cooling. Seven-μm-thick serial sections were cut using a rotating microtome from the TMA blocks and placed on SuperFrost® Plus-slides.

Immunohistochemistry

Seven-μm-thick sections were deparaffinized and rehydrated according to the standard procedure. The TMA sections with the postmortem core samples and the surgical biopsy were stained employing antibodies as described in Table 3 and the following antigen retrieval methods were applied: 80% formic acid for 2, 10, 60 min and for 6 h or microwave in citrate buffer pH 6.0 3 × 5 min or a combination of microwave in citrate buffer pH 6.0 3 × 5 min and 80% formic acid for 10 min. Following the pretreatment, the sections were incubated with normal goat serum for 30 min at room temperature to block non-specific reactions. After the epitope unmasking, antibodies were applied in the dilution described in Table 3. Then the sections were incubated overnight at 4 °C. On the following day, the sections were incubated with the biotinylated second antibody for 30 min followed by streptavidin enzyme conjugate (LABSA Zymed laboratories, South San Francisco, CA) for 30 min at room temperature. The reaction product was visualized using romulin 3-amino-9-ethyl carbazole (AEC). All immunostained sections were counterstained with Harris' haematoxylin, dehydrated, and mounted in DePex. To confirm the colocalization of positive labeling, serial sections were stained by applying the most optimal staining protocol (Table 3).

The staining results were evaluated under light microscopy. Extracellular and intracellular IR was assessed in each core and the extracellular IR was designated as being present or not. The intracellular IR, which was seen as grainy labeling in the cytoplasm, was noted as seen or not seen. In the serial sections, the colocalization was assessed under light microscopy at low and high magnifications.

RESULTS

Extracellular labeling

All commercial antibodies directed to the amino acid residues present in Aβ (82E1, 6E10, 6F3D, 4G8, $A\beta_{40}$, $A\beta_{42}$, 12F4) stained extracellular amyloid deposits irrespective of the antigen retrieval method. Extracellular aggregates were seen in the TMAa in cases #6, 8, 10, and 11, and as expected, in the TMAd in all cases of AD. The fleecy/diffuse aggregates were seen to the same extent independent of which commercial Aβ antibody was used. Contrary to the above, antibodies against AβPP or its derivatives (AβPP C-terminal, AβPP40.10) did not label the extracellular aggregates.

Formic acid treatment enhanced the intensity of extracellular IR and the contrast increased with up to 60 min of formic acid pretreatment. The combined pretreatment, i.e., boiling in a microwave oven 3 × 5 min in citrate buffer and incubation in 80% formic acid for 10 min, intensified the extent of eAβ labeling even further, however, the concomitant increase in the background IR decreased the contrast. Intense labeling of amyloid deposits, after boiling in a microwave oven and incubation in formic acid, led to the impression that there was an increased number of compact and a decreased number of diffuse deposits. If the antigen retrieval method was restricted to only boiling in a microwave oven this led to an overall weaker IR staining of the extracellular deposits.

Intracellular labeling

Intracellular labeling was observed with antibodies against AβPP or its derivatives in all scores in the TMAa and TMAd regardless of the antigen retrieval method. With three of seven antibodies directed to the amino acid residues present in Aβ (82E1, 6E10, 4G8), an obvious granular intracellular labeling was seen in all scores in the TMAa and TMAd and the labeling was present in cells already at the age of 2 and the IR remained high in all samples over entire age range of 2 to 100 years. The labeled grains were small and coarse and located close to the nucleus. With antibodies $A\beta_{40}$, $A\beta_{42}$, and 12F4, intracellular labeling was only detected in the TMAd in cores taken from the temporal cortex of AD cases ($n = 7$). The monoclonal antibody 6F3D did not display intracellular IR in any of the cores in the TMAa or TMAd. The mode of death did not seem to alter the amount of intracellular IR.

Antibodies directed to the N-terminus or mid portion of Aβ

The monoclonal antibody against N-terminus of Aβ, 6F3D, did not display intracellular IR regardless of which antigen retrieval method was used in any of the cores in the TMAa or TMAd. Contrary to the above, with monoclonal antibody directed to the mid-portion of Aβ, clone 4G8, intracellular labeling was seen in all TMAa and TMAd cores irrespective of age, disease state, or antigen retrieval method. However, a more intense intracellular labeling was detected in sections pretreated with heating as compared to those pretreated with formic acid. The intracellular labeling with N-terminal monoclonal antibody 6E10 was detected in the cores in TMAa and TMAd only when the combined pretreatment, i.e., boiling in the microwave oven and incubation in formic acid was used. In line with the above, when the N-terminal monoclonal antibody clone 82E1 was used, then intracellular labeling was noted with the high magnification in most cores in the TMAa (Fig. 2) and in the TMAd in two AD cases (#13, 15), and in three FTLD-TDP43 cases (#21, 22, 25) but only when the combination of heat and formic acid pretreatment was used. While applying clone 82E1, a few cells with labeling were noted in TMAa in the 2-year old child at the x200 magnification, whereas in the oldest case with additional eAβ aggregates, iAβ labeling was seen in almost every cell. To summarize, the combination of heat and formic acid was essential to visualize the intracellular staining with antibodies such as 6E10 and 82E1, whereas the intracellular staining with monoclonal antibody 4G8 was seen regardless of antigen retrieval method.

When comparing the images of serial sections and assessing neurons with identifiable nuclei, intracellular labeling was noted in the same cell with antibodies 4G8 or 6E10 and AβPP 22C11 (Fig. 3).

Antibodies directed to the C-terminus of Aβ

The intracellular staining with the polyclonal antibodies directed to the neoepitopes, $A\beta_{40}$ and $A\beta_{42}$, and the monoclonal antibody $A\beta_{42}$, was not detected in the cores in TMAa, even though four of the TMAa core samples displayed additional eAβ (#6, 8, 10, and 11). Interestingly in the TMAd, intracellular labeling was seen with both mono- and polyclonal Aβ antibodies in all cores taken from the temporal cortex of AD cases ($n = 7$) displaying eAβ aggregates, most being in Braak stages V to VI (Table 2, Fig. 4). The labeling

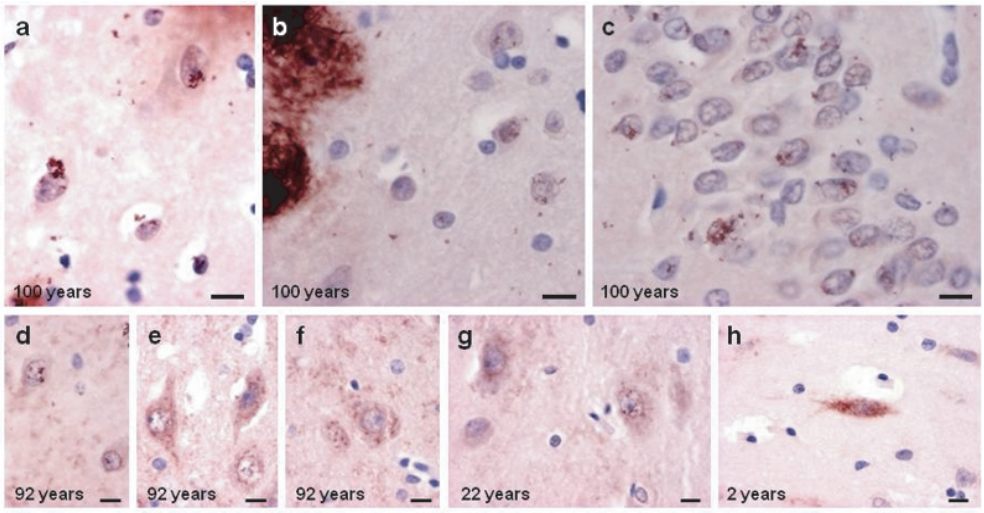

Fig. 2. Photomicrographs displaying the 82E1 positive grainy perinuclear/intracellular labeling seen in the hippocampal cells in a 100 year old subject (a-c), in a 92 year old subject (d-f), in a 22 year old subjects (g) and in the 2 year old child (h). Scale bar = 10 μm.

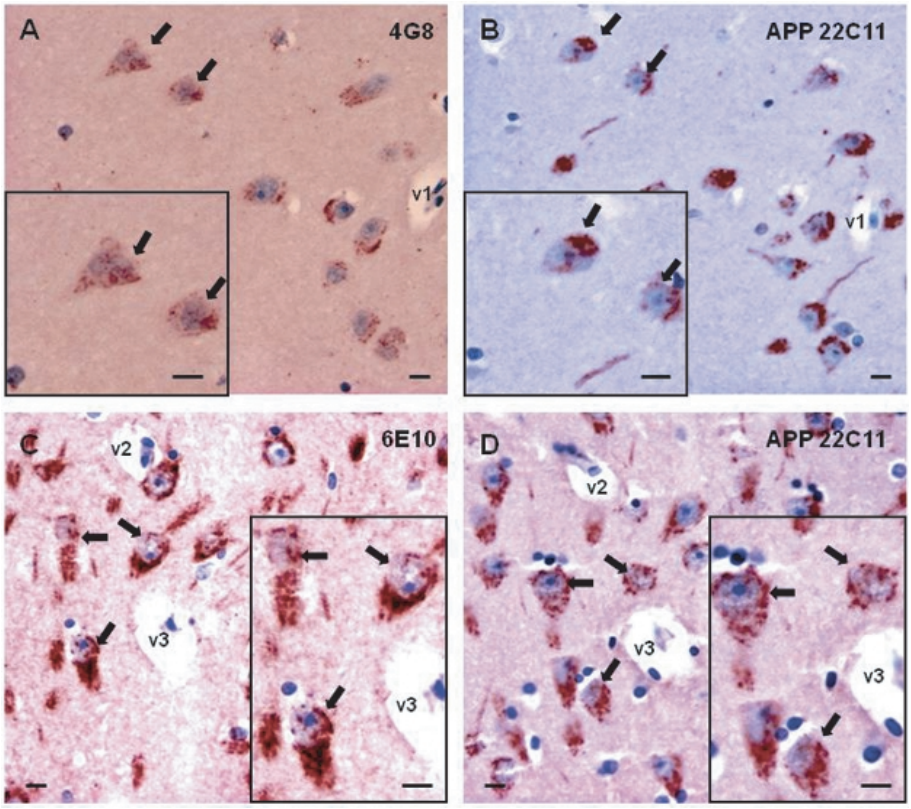

Fig. 3. Application of clones 4G8, 6E10 and AβPP 22C11 on serial sections. 4G8 and 6E10 positive neurons (a, c) bisected during sectioning (arrows, enlarged in insets) were identified in the adjacent AβPP-stained sections (b, d) using nearby vessels (v1-3) as landmarks. Scale bars = 10 μm.

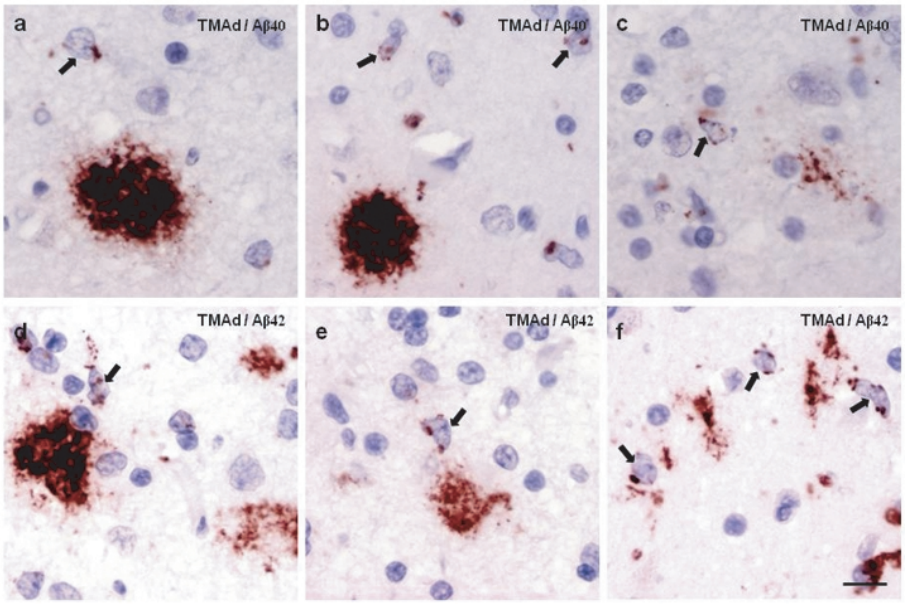

Fig. 4. The intracellular labeling of Aβ in the TMA block of disease with the polyclonal antibodies Aβ_{40} and Aβ_{42}. Note a grainy perinuclear/intracellular labeling (neurons or glial cells) in core samples where in addition extracellular Aβ IR aggregates were seen. Scale bar $= 10$ μm.

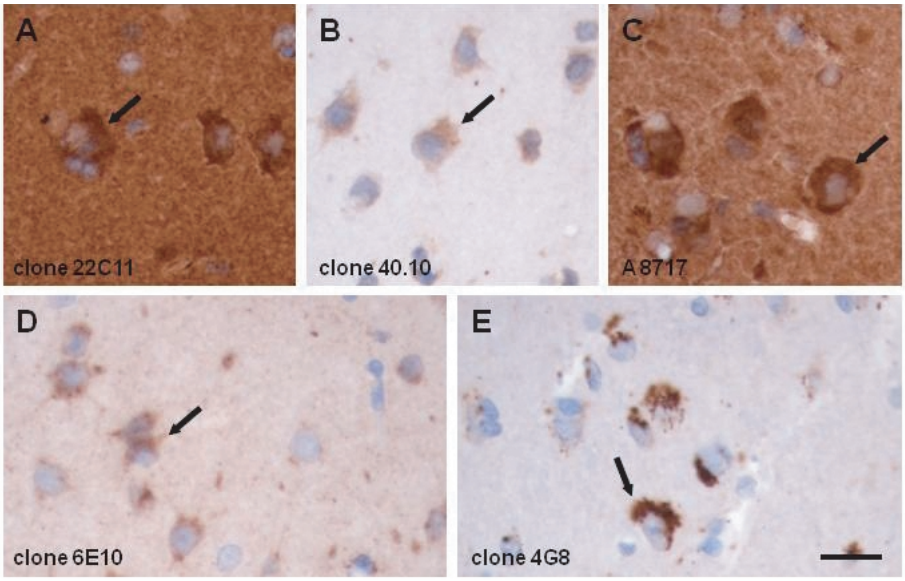

Fig. 5. A brain biopsy obtained from frontal cortex from a 75 year-old female was immunohistochemically stained while applying antibodies as given in the Fig. 1 and Table 3. Note that with all antibodies intracytoplasmic perinuclear grainy labeling was seen. Some antibodies directed to AβPP also strongly labeled the neuropil.

was seen at the high magnification and based on the cytological features; the cells were either neurons or glial cells. It is noteworthy that some labeled grains were also seen in the neuropil complicating the interpretation of the staining results. These results were obtained regardless of which of the following antigen retrieval method was used: 80% formic acid, or microwave in citrate buffer pH 6.0 3 × 5 min, or a combination of microwave in citrate buffer pH 6.0 3 × 5 min and 80% formic acid for 10 min.

Surgical sample

In the surgical biopsy, no eAβ aggregates were seen with any of the applied antibodies. Intracellular labeling was seen with antibodies 6E10, 4G8, 82E1, AβPP22C11, AβPP 40.10, and AβPP C-terminus (Fig. 5). No IR was seen when applying mono- or polyclonal C-terminal Aβ antibodies.

DISCUSSION

In the present study, the eAβ IR was repeatedly detected with antibodies directed to the amino acid residues present in the Aβ, whereas no extracellular IR was noted with antibodies directed to the AβPP or its derivatives. This finding confirms earlier reports that extracellular aggregates consist of Aβ rather than AβPP. In contrast to the clear results with respect to the extracellular labeling, the staining results regarding the iAβ were variable and dependent on both the chosen antibody and the antigen retrieval method.

Seven different commercial antibodies (4G8, 6E10, 82E1, 6F3D, $A\beta_{40}$, $A\beta_{42}$, 12F4) directed to the N- or C-terminus or mid-portion of the Aβ fragment were used in the present study. All of these antibodies have been claimed to recognize Aβ in the tissue according to their specifications. With three (4G8, 6E10, 82E1) of these seven antibodies, intracellular IR was detected in all cases in the cores in TMAa and TMAd independent of disease state, and it is noteworthy that the IR was already seen at the age of two years. Contrary to the above, with the C-terminal antibodies, i.e., $A\beta_{40}$, $A\beta_{42}$, and 12F4, the iAβ IR was only detected in the AD cases. Interestingly, monoclonal antibody 6F3D did not label the intracellular compartment with any of the used techniques in any of the cores. Hence, if only the antibodies against N-terminus or the mid-portion of the Aβ had been applied, the conclusion would have been that the iAβ is seen in all cases from 2 year old up to 100 year old irrespective of condition. This is in agreement with reports claiming that the iAβ represents a product of normal neuronal metabolism [23, 25,34]. On the contrary, if only the antibodies directed to C-terminus of the Aβ had been used, the conclusion would have been that the iAβ is only detected in the brains of subjects with AD, and this would confirm the reports suggesting that the accumulation of iAβ is an event associated with the pathogenesis of AD [4,7,11, 13,17,45]. These results emphasize that the obtained staining results and their interpretation are strongly dependent on the choice of antibody and thus, it has to be concluded that indeed multiple antibodies, including antibodies directed to the N- and C-terminal neoepitopes, must be used when assessing the presence of iAβ [46].

In the present study, in all cases (TMAa and TMAd) independent of disease state or age intracellular staining was seen with the antibodies 4G8, 6E10, and 82E1. The assessment of mirror images of consecutive sections revealed that not only the Aβ clones 4G8 and 6E10 but also the AβPP22C11 labeled the cytoplasm of the same cell. In 1996, LeBlanc and co-workers demonstrated that the clone 4G8 immunoprecipitated not only Aβ but also the full-length AβPP and AβPP C-terminal fragment [47]. This finding was further confirmed in 2002 by Takahashi and colleagues who reported that clone 4G8 reacted with AβPP C-terminal fragments in Western blotting [34]. The antibodies directed close to the N-terminus may theoretically also recognize Aβ that is still embedded in the membrane. The amino acid sequence that is recognized by clones 4G8 and 6E10 is also found in the full-length AβPP and its derivatives, hence these antibodies are unable to differentiate Aβ from AβPP [32,33,47,48]. The clone 82E1 that detects fragments generated by β-secretase cleavage [48] labels both soluble and insoluble Aβ to a similar degree and recognizes the free N-terminus of the AβPP C99 fragment and this C99 fragment is then later cleaved by the γ-secretase to generate Aβ. Therefore, the detection of intracellular IR with clone 82E1 only indicates that the cleavage product of β-secretase is present in the intracellular compartment. The commonly used antibodies such as 4G8, 6E10, and 82E1 fail to distinguish Aβ from AβPP if examined by IHC. Thus, one must question the validity of reports claiming that it is Aβ rather than AβPP that is present in the intracellular compartment if antibodies such as 4G8, 6E10, and 82E1 have been utilized (Table 1).

The accumulation of iAβ has also been reported while applying commercial antibodies directed to the

C-terminus of Aβ (neoepitope) that have been reported to be specific to Aβ [3,4,7–9,11,13,15–17,19,22,23, 39]. In the present study, while applying commercial antibodies directed to the C-terminus of Aβ, an intense eAβ IR (positive control) was seen in the TMAa and TMAd core samples. In the TMAa, no iAβ was detected in any of the cores irrespective of whether or not additional eAβ was seen. Our negative result, i.e., lack of intracellular labeling while applying antibodies directed to $A\beta_{40}$ and $A\beta_{42}$ is confirmation of the reports of Kuo and co-workers and Mochizuki and coworkers [15,49]. Contrary to the above, in all subjects with AD, intracellular IR was indeed detected with the antibodies directed to C-terminal neoepitopes $A\beta_{40}$ and $A\beta_{42}$. The presence of iAβ in subjects with additional eAβ aggregates and widespread neuronal degeneration (Braak stages IV to VI) and the absence of iAβ in subjects without additional eAβ aggregates does suggest that it is the internalization of Aβ from the extracellular pool rather than the intracellular production of Aβ is accelerated. This finding is in line with several previous studies reporting the presence of iAβ in the AD brains [3,4,7–9,11,15,17,19,22,23,39]. In 2008, Aoki and colleagues, while using laser capture micro dissection to isolate neurons of hippocampus, noted $iA\beta_{42}$ in all investigated subjects [50]. They detected a significant increase in the level of $iA\beta_{42}$ in AD subjects when compared with controls (mean age ranging from 71 to 90) and the highest levels were seen in subjects with familial disease [50]. Thus, the lack of IR in the brains obtained from subjects without neuronal degeneration may indicate that the level of the iAβ is very low, undetectable at the light microscopic level by IHC methods. In conclusion, our and previous results indicate that iAβ labeling with the specific antibodies can indeed be seen at the light microscopy level and this is associated with eAβ labeling but only in tissue samples obtained from diseased brains with widespread neuronal degeneration.

The antigen retrieval method, re-exposing and reshaping the critical epitopes, is significant as is shown in this study while applying clones 6E10 and 82E1 [5, 19,21,37–39]. Formic acid is a widely used antigen retrieval method to enhance the IR of eAβ, whereas the effect of formic acid pretreatment on the iAβ is less clear. In the present study, the intracellular IR with clones 6E10 and 82E1 was only seen when the sections were both heated and incubated in formic acid indicating that heat pretreatment was essential for the staining of iAβ with these antibodies. This result is in line with previous publications stating that formic acid pretreatment alone is not sufficient to visualize the intracellular IR [19,39]. In the recently published study of Christensen and colleagues, it was demonstrated that heat alone increases the cross-reaction at least of clone 4G8 and AβPP [5], i.e., this heat-induced cross-reaction may explain the more intense intracellular IR with clone 4G8. This result corroborates that it is AβPP rather than Aβ that is present in the intracellular compartment with antibody such as 4G8. The other antibody directed close to the N-terminus of the Aβ, clone 6F3D, repeatedly failed to label the intracellular compartment even though the extracellular labeling of aggregates was excellent. As far as we are aware, no intracellular labeling with clone 6F3D has been so far detected. Whether this is due to poor preservation of the epitope during pre-sectioning treatment, the spatial configuration of the protein, or the localization of the epitopes within the protein, is currently unknown [40, 51].

In addition to the antibody and the antigen retrieval method, species differences may also influence on the staining results [35,36]. All widely used transgenic mice models are based on AβPP mutations that are known to influence the production and aggregation of $A\beta_{42}$ peptide and thus Aβ peptides in transgenic mice models are in a higher aggregation state when compared to humans [5]. Moreover, it has been shown *in vivo* that transcription factors responsible for gene expression diverge between human and mouse [52]. Lately the epigenetic alterations have been linked to the development of neurological disorders such as AD and Parkinson disease [53]. It can be expected that like transcription factors also the epigenetic factors, DNA methylation and histone modifications, diverge between human and animals. These physiological differences affect all studies that use the mouse as a model for human biology [52]. Therefore, the results obtained from animal studies are not as such directly comparable with results obtained while studying brain tissue obtained from humans.

As shown in the present study, the obtained staining results are strongly dependent on the applied antibody, antigen retrieval method, and the tissue characteristics. Furthermore, it should be noted that the interpretation of the results while applying one and the same antibody has been contradictory, i.e., in one study the IR has been interpreted as representing Aβ and in another as AβPP [3,32,33]. Summarizing all the available reports (Table 1) and our own results, we conclude that at the light microscopic level it is not possible to differentiate Aβ from AβPP, when monoclonal antibodies such

as 4G8, 6E10, and 82E1 have been used. In routine diagnostics while assessing the extracellular accumulation of Aβ, these antibodies are commendable, but they are not useful if one is trying to investigate the intracellular accumulation of Aβ. When the goal is to assess Aβ protein, and to determine whether protein is localized in the intracellular space, then antibody directed to neoepitopes that recognize the site of terminal sequence of Aβ and which are able to differentiate Aβ from AβPP should be used. In conclusion, if one wishes to unambiguously confirm the presence or absence of a protein in tissue, multiple antibodies directed to several different amino acid sequences should be used and the significant influence of antigen retrieval methods should also always be considered.

ACKNOWLEDGMENTS

We thank Erkki Kuusisto, PhD, Mrs. Tarja Kauppinen, Mrs. Merja Fali, Mr. Heikki Luukkonen, and Mr. Hannu Tiainen for their valuable help. This study was supported by European Union grant FP6: BNEII No LSHM-CT-2004-503039, the Finnish Cultural Foundation, Health Research Council of the Academy of Finland, EVO grant 5772708 of Kuopio University Hospital, and the Nordic Centre of Excellence of Neurodegeneration. This article reflects only the authors' views and that the Community is not liable for any use that may be made of the information contained therein. The study has been authorized by the Ethics Committee of Kuopio University Hospital. Source of support: EU grant FP6: BNEII No LSHM-CT-2004-503039.

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=303).

REFERENCES

[1] Grundke-Iqbal I, Iqbal K, George L, Tung YC, Kim KS, Wisniewski HM (1989) Amyloid protein and neurofibrillary tangles coexist in the same neuron in Alzheimer disease. *Proc Natl Acad Sci U S A* **86**, 2853-2857.

[2] Achim CL, Adame A, Dumaop W, Everall IP, Masliah E, Neurobehavioral Research Center (2009) Increased accumulation of intraneuronal amyloid beta in HIV-infected patients. *J Neuroimmune Pharmacol* **4**, 190-199.

[3] Akiyama H, Mori H, Saido T, Kondo H, Ikeda K, McGeer PL (1999) Occurrence of the diffuse amyloid beta-protein (Abeta) deposits with numerous Abeta-containing glial cells in the cerebral cortex of patients with Alzheimer's disease. *Glia* **25**, 324-331.

[4] Cataldo AM, Petanceska S, Terio NB, Peterhoff CM, Durham R, Mercken M, Mehta PD, Buxbaum J, Haroutunian V, Nixon RA (2004) Abeta localization in abnormal endosomes: association with earliest Abeta elevations in AD and Down syndrome. *Neurobiol Aging* **25**, 1263-1272.

[5] Christensen DZ, Bayer TA, Wirths O (2009) Formic acid is essential for immunohistochemical detection of aggregated intraneuronal Abeta peptides in mouse models of Alzheimer's disease. *Brain Res* **1301**, 116-125.

[6] Cruz JC, Kim D, Moy LY, Dobbin MM, Sun X, Bronson RT, Tsai LH (2006) P25/cyclin-Dependent Kinase 5 Induces Production and Intraneuronal Accumulation of Amyloid Beta *in Vivo*. *J Neurosci* **26**, 10536-10541.

[7] D'Andrea MR, Peterson PA, Lee DH, Nagele RG, Wang HY (2001) Evidence that neurones accumulating amyloid can undergo lysis to form amyloid plaques in Alzheimer's disease. *Histopathology* **38**, 120-134.

[8] D'Andrea MR, Nagele RG, Gumula NA, Reiser PA, Polkovitch DA, Hertzog BM, Andrade-Gordon P (2002) Lipofuscin and Aβ42 exhibit distinct distribution patterns in normal and Alzheimer's disease brains. *Neurosci Lett* **323**, 45-49.

[9] D'Andrea MR, Lee DHS, Nagele RG, Wang H (2002) Consistent immunohistochemical detection of intracellular beta-amyloid42 in pyramidal neurons of Alzheimer's disease entorhinal cortex. *Neurosci Lett* **333**, 163-166.

[10] Gomez-Ramos P, Asuncion Moran M (2007) Ultrastructural localization of intraneuronal Abeta-peptide in Alzheimer disease brains. *J Alzheimers Dis* **11**, 53-59.

[11] 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 Abeta42 accumulation in human brain. *Am J Pathol* **156**, 15-20.

[12] Green DA, Masliah E, Vinters HV, Beizai P, Moore DJ, Achim CL (2005) Brain deposition of beta-amyloid is a common pathologic feature in HIV positive patients. *AIDS* **19**, 407-411.

[13] Gyure KA, Durham R, Stewart WF, Smialek JE, Troncoso JC (2001) Intraneuronal abeta-amyloid precedes development of amyloid plaques in Down syndrome. *Arch Pathol Lab Med* **125**, 489-492.

[14] LaFerla FM, Troncoso JC, Strickland DK, Kawas CH, Jay G (1997) Neuronal cell death in Alzheimer's disease correlates with apoE uptake and intracellular Abeta stabilization. *J Clin Invest* **100**, 310-320.

[15] Mochizuki A, Tamaoka A, Shimohata A, Komatsuzaki Y, Shoji S (2000) Abeta42-positive non-pyramidal neurons around amyloid plaques in Alzheimer's disease. *Lancet* **355**, 42-43.

[16] Mori C, Wisniewski TM, Tolan DR, Yamaguch H, Spooner ET, Wisniewsk KE, Lemere CA, Saido TC, Selkoe DJ (2002) Intraneuronal Abeta42 accumulation in Down syndrome brain. *Amyloid* **9**, 88-102.

[17] Nagele RG, D'Andrea MR, Anderson WJ, Wang HY (2002) Intracellular accumulation of beta-amyloid(1-42) in neurons is facilitated by the alpha 7 nicotinic acetylcholine receptor in Alzheimer's disease. *Neuroscience* **110**, 199-211.

[18] Oakley H, Cole SL, Logan S, Maus E, Shao P, Craft J, Guillozet-Bongaarts A, Ohno M, Disterhoft J, Van Eldik L, Berry R, Vassar R (2006) Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. *J Neurosci* **26**, 10129-10140.

[19] Ohyagi Y, Tsuruta Y, Motomura K, Miyoshi K, Kikuchi H, Iwaki T, Taniwaki T, Kira J (2007) Intraneuronal amyloid

beta42 enhanced by heating but counteracted by formic acid. *J Neurosci Methods* **159**, 134-138.

[20] Ramage SN, Anthony IC, Carnie FW, Busuttil A, Robertson R, Bell JE (2005) Hyperphosphorylated tau and amyloid precursor protein deposition is increased in the brains of young drug abusers. *Neuropathol Appl Neurobiol* **31**, 439-448.

[21] Sheng JG, Bora SH, Xu G, Borchelt DR, Price DL, Koliatsos VE (2003) Lipopolysaccharide-induced-neuroinflammation increases intracellular accumulation of amyloid precursor protein and amyloid beta peptide in APPswe transgenic mice. *Neurobiol Dis* **14**, 133-145.

[22] Wang HY, D'Andrea MR, Nagele RG (2002) Cerebellar diffuse amyloid plaques are derived from dendritic Abeta42 accumulations in Purkinje cells. *Neurobiol Aging* **23**, 213-223.

[23] Wegiel J, Kuchna I, Nowicki K, Frackowiak J, Mazur-Kolecka B, Imaki H, Wegiel J, Mehta PD, Silverman WP, Reisberg B, Deleon M, Wisniewski T, Pirttilla T, Frey H, Lehtimaki T, Kivimaki T, Visser FE, Kamphorst W, Potempska A, Bolton D, Currie JR, Miller DL (2007) Intraneuronal Abeta immunoreactivity is not a predictor of brain amyloidosis-beta or neurofibrillary degeneration. *Acta Neuropathol* **113**, 389-402.

[24] Glenner GG, Wong CW (1984) Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein. *Biochem Biophys Res Commun* **122**, 1131-1135.

[25] Haass C, Schlossmacher MG, Hung AY, Vigo-Pelfrey C, Mellon A, Ostaszewski BL, Lieberburg I, Koo EH, Schenk D, Teplow DB (1992) Amyloid beta-peptide is produced by cultured cells during normal metabolism. *Nature* **359**, 322-325.

[26] Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K (1985) Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc Natl Acad Sci U S A* **82**, 4245-4249.

[27] Miller DL, Papayannopoulos IA, Styles J, Bobin SA, Lin YY, Biemann K, Iqbal K (1993) Peptide compositions of the cerebrovascular and senile plaque core amyloid deposits of Alzheimer's disease. *Arch Biochem Biophys* **301**, 41-52.

[28] Tanzi RE, McClatchey AI, Lamperti ED, Villa-Komaroff L, Gusella JF, Neve RL (1988) Protease inhibitor domain encoded by an amyloid protein precursor mRNA associated with Alzheimer's disease. *Nature* **331**, 528-530.

[29] Kinoshita A, Fukumoto H, Shah T, Whelan CM, Irizarry MC, Hyman BT (2003) Demonstration by FRET of BACE interaction with the amyloid precursor protein at the cell surface and in early endosomes. *J Cell Sci* **116**, 3339-3346.

[30] Xu H, Greengard P, Gandy S (1995) Regulated formation of Golgi secretory vesicles containing Alzheimer beta-amyloid precursor protein. *J Biol Chem* **270**, 23243-23245.

[31] Mizuguchi M, Ikeda K, Kim SU (1992) Differential distribution of cellular forms of beta-amyloid precursor protein in murine glial cell cultures. *Brain Res* **584**, 219-225.

[32] Knobloch M, Konietzko U, Krebs DC, Nitsch RM (2007) Intracellular Abeta and cognitive deficits precede beta-amyloid deposition in transgenic arcAbeta mice. *Neurobiol Aging* **28**, 1297-1306.

[33] Lord A, Kalimo H, Eckman C, Zhang XQ, Lannfelt L, Nilsson LN (2006) The Arctic Alzheimer mutation facilitates early intraneuronal Abeta aggregation and senile plaque formation in transgenic mice. *Neurobiol Aging* **27**, 67-77.

[34] Takahashi RH, Milner TA, Li F, Nam EE, Edgar MA, Yamaguchi H, Beal MF, Xu H, Greengard P, Gouras GK (2002) Intraneuronal Alzheimer abeta42 accumulates in multivesicular bodies and is associated with synaptic pathology. *Am J Pathol* **161**, 1869-1879.

[35] Harding AJ, Wong A, Svoboda M, Kril JJ, Halliday GM (1997) Chronic alcohol consumption does not cause hippocampal neuron loss in humans. *Hippocampus* **7**, 78-87.

[36] Mandavilli A (2006) The amyloid code. *Nat Med* **12**, 747-751.

[37] Alafuzoff I, Pikkarainen M, Arzberger T, Thal DR, Al-Sarraj S, Bell J, Bodi I, Budka H, Capetillo-Zarate E, Ferrer I, Gelpi E, Gentleman S, Giaccone G, Kavantzas N, King A, Korkolopoulou P, Kovacs GG, Meyronet D, Monoranu C, Parchi P, Patsouris E, Roggendorf W, Stadelmann C, Streichenberger N, Tagliavini F, Kretzschmar H (2008) Inter-laboratory comparison of neuropathological assessments of beta-amyloid protein: a study of the BrainNet Europe consortium. *Acta Neuropathol* **115**, 533-546.

[38] Beach TG, Sue LI, Walker DG, Roher AE, Lue L, Vedders L, Connor DJ, Sabbagh MN, Rogers J (2008) The Sun Health Research Institute Brain Donation Program: description and experience, 1987-2007. *Cell Tissue Bank* **9**, 229-245.

[39] D'Andrea MR, Reiser PA, Polkovitch DA, Gumula NA, Branchide B, Hertzog BM, Schmidheiser D, Belkowski S, Gastard MC, Andrade-Gordon P (2003) The use of formic acid to embellish amyloid plaque detection in Alzheimer's disease tissues misguides key observations. *Neurosci Lett* **342**, 114-118.

[40] Shi SR, Cote RJ, Taylor CR (1997) Antigen retrieval immunohistochemistry: past, present, and future. *J Histochem Cytochem* **45**, 327-343.

[41] Hynd MR, Lewohl JM, Scott HL, Dodd PR (2003) Biochemical and molecular studies using human autopsy brain tissue. *J Neurochem* **85**, 543-562.

[42] Alafuzoff I, Arzberger T, Al-Sarraj S, Bodi I, Bogdanovic N, Braak H, Bugiani O, Del-Tredici K, Ferrer I, Gelpi E, Giaccone G, Graeber MB, Ince P, Kamphorst W, King A, Korkolopoulou P, Kovacs GG, Larionov S, Meyronet D, Monoranu C, Parchi P, Patsouris E, Roggendorf W, Seilhean D, Tagliavini F, Stadelmann C, Streichenberger N, Thal DR, Wharton SB, Kretzschmar H (2008) Staging of neurofibrillary pathology in Alzheimer's disease: a study of the BrainNet Europe Consortium. *Brain Pathol* **18**, 484-496.

[43] Kauppinen T, Martikainen P, Alafuzoff I (2006) Human postmortem brain tissue and 2-mm tissue microarrays. *Appl Immunohistochem Mol Morphol* **14**, 353-359.

[44] Martikainen P, Louhelainen AM, Kauppinen T, Alafuzoff I (2006) Human brain tissue microarrays as a platform to investigate diseases of the nervous system. *Brain Res* **1089**, 33-43.

[45] Wirths O, Multhaup G, Bayer TA (2004) A modified beta-amyloid hypothesis: intraneuronal accumulation of the beta-amyloid peptide–the first step of a fatal cascade. *J Neurochem* **91**, 513-520.

[46] LaFerla FM, LaFerla FM, Green KN, Oddo S (2007) Intracellular amyloid-β in Alzheimer's disease. *Nat Rev Neurosci* **8**, 499.

[47] LeBlanc AC, Xue R, Gambetti P (1996) Amyloid precursor protein metabolism in primary cell cultures of neurons, astrocytes, and microglia. *J Neurochem* **66**, 2300-2310.

[48] Horikoshi Y, Sakaguchi G, Becker AG, Gray AJ, Duff K, Aisen PS, Yamaguchi H, Maeda M, Kinoshita N, Matsuoka Y (2004) Development of Abeta terminal end-specific antibodies and sensitive ELISA for Abeta variant. *Biochem Biophys Res Commun* **319**, 733-737.

[49] Kuo YM, Beach TG, Sue LI, Scott S, Layne KJ, Kokjohn TA, Kalback WM, Luehrs DC, Vishnivetskaya TA, Abramowski D, Sturchler-Pierrat C, Staufenbiel M, Weller RO, Roher AE (2001) The evolution of A beta peptide burden in the

APP23 transgenic mice: implications for A beta deposition in Alzheimer disease. *Mol Med* **7**, 609-618.

[50] Aoki M, Volkmann I, Tjernberg LO, Winblad B, Bogdanovic N (2008) Amyloid beta-peptide levels in laser capture microdissected cornu ammonis 1 pyramidal neurons of Alzheimer's brain. *Neuroreport* **19**, 1085-1089.

[51] Kayed R, Head E, Sarsoza F, Saing T, Cotman CW, Necula M, Margol L, Wu J, Breydo L, Thompson JL, Rasool S, Gurlo T, Butler P, Glabe CG (2007) Fibril specific, conformation dependent antibodies recognize a generic epitope common to amyloid fibrils and fibrillar oligomers that is absent in prefibrillar oligomers. *Mol Neurodegener* **2**, 18.

[52] Odom DT, Dowell RD, Jacobsen ES, Gordon W, Danford TW, MacIsaac KD, Rolfe PA, Conboy CM, Gifford DK, Fraenkel E (2007) Tissue-specific transcriptional regulation has diverged significantly between human and mouse. *Nat Genet* **39**, 730-732.

[53] Urdinguio RG, Sanchez-Mut JV, Esteller M (2009) Epigenetic mechanisms in neurological diseases: genes, syndromes, and therapies. *Lancet Neurol* **8**, 1056-1072.

[54] Wegiel J (2002) Intraneuronal accumulation of N-terminally truncated amyloid beta. *J Neuropathol Exp Neurol* **61**, 483-483.

[55] Van Broeck B, Vanhoutte G, Pirici D, Van Dam D, Wils H, Cuijt I, Vennekens K, (2008), Theuns J, (2008) Intraneuronal amyloid β and reduced brain volume in a novel APP T714I mouse model for Alzheimer's disease. *Neurobiol Aging* **2**, 241-252.

[56] Kim KS, Wen GY, Bancher C, Chen CMJ, Sapienza VJ, Hong H, Wisniewski HM (1990) Detection and quantitation of amyloid beta peptide with two monoclonal antibodies. *Neurosci Res Commun*, 113-122.

[57] Wang R, Sweeney D, Gandy SE, Sisodia SS (1996) The profile of soluble amyloid beta protein in cultured cell media. Detection and quantification of amyloid beta protein and variants by immunoprecipitation-mass spectrometry. *J Biol Chem* **271**, 31894-31902.

[58] 58. Matsunaga Y, Saito N, Fujii A, Yokotani J, Takakura T, Nishimura T, Esaki H, Yamada T (2002) A pH-dependent conformational transition of Abeta peptide and physicochemical properties of the conformers in the glial cell. *Biochem J* **361**, 547-556.