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ORIGINAL PAPER

Accumulation of intraneuronal Aβ correlates with ApoE4 genotype

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Abstract In contrast to extracellular plaque and intracellular tangle pathology, the presence and relevance of intraneuronal Aβ in Alzheimer's disease (AD) is still a matter of debate. Human brain tissue offers technical challenges such as post-mortem delay and uneven or prolonged tissue fixation that might affect immunohistochemical staining. In addition, previous studies on intracellular Aβ accumulation in human brain often used antibodies targeting the C-terminus of Aβ and differed strongly in the pretreatments used. To overcome these inconsistencies, we performed extensive parametrical testing using a highly specific N-terminal Aβ antibody detecting the aspartate at position 1, before developing an optimal staining protocol for intraneuronal Aβ detection in paraffin-embedded sections from AD patients. To rule out that this antibody also detects the β-cleaved APP C-terminal fragment (β-CTF, C99) bearing the same epitope, paraffin-sections of transgenic mice overexpressing the C99-fragment were stained without any evidence for cross-reactivity in our staining protocol. The staining intensity of intraneuronal Aβ in cortex and hippocampal tissue of 10 controls and 20 sporadic AD cases was then correlated to patient data including sex, Braak stage, plaque load, and apolipoprotein E (ApoE) genotype. In particular, the presence of one or two ApoE4 alleles strongly correlated with an increased accumulation of intraneuronal Aβ peptides. Given that ApoE4 is a major genetic risk factor for AD and is involved in neuronal cholesterol transport, it is tempting to speculate that perturbed intracellular trafficking is involved in the increased intraneuronal Aβ aggregation in AD.

Keywords Intracellular Abeta · Intraneuronal Abeta · Alzheimer · Microwave · Amyloid · Astrocyte · ApoE · Formic acid · Heat · Antigen retrieval

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Introduction

Genetic studies on familial Alzheimer's disease (AD) patients have revealed mutations in genes linked to the beta amyloid (Aβ) generating cascade. Although this provides strong support for the amyloid hypothesis as the underlying pathological mechanism of AD [30], some controversies remain to be resolved. For instance, the accumulation of extracellular Aβ plaques does not correlate with the cognitive decline observed in AD patients [1]. In addition, a massive loss of neurons around plaques is not seen in human brain [54] and only rarely observed after the onset of Aβ plaque pathology in various different mouse models [22, 24].

One possible explanation is that, rather than extracellular Aβ plaque deposition, accumulation of Aβ inside neurons could be driving AD pathology, and may eventually lead to intracellular deficits in function and neuronal loss [25, 37, 68]. Intraneuronal Aβ has been reported to disrupt fast axonal transport (FAT) in isolated axoplasms [50], to impair multi vesicular body (MVB) sorting by inhibition of the ubiquitin–proteasome system [2] and to induce synaptic dysfunction leading to reduced PSD-95 and consequently GluR1 levels in synapses [3].

The accumulation of intraneuronal Aβ peptides in AD brain has been sporadically reported since the late 1980s [27]. However, initial problems with the inability of the antibodies to separate between full length amyloid precursor protein (APP) and Aβ itself founded a skepticism toward the presence of intraneuronal Aβ that has been difficult to eliminate [25, 37]. Despite the initial technical complications, several studies using Aβ40/42 end-specific antibodies have later reported the presence of intraneuronal Aβ in AD and healthy controls [17–19, 23, 26, 44, 46, 65], as well as in Down syndrome patients who are known to develop AD at an early age [29, 45]. One study isolated human hippocampal pyramidal neurons from the CA1 of AD patients by laser capture microdissection, determined Aβ peptide levels using ELISA quantification and reported an increased intraneuronal Aβ42/Aβ40 ratio in AD patients compared to controls [4]. Still, the presence and impact of intraneuronal Aβ in human AD tissue is a matter of controversial debate [22].

The data on intraneuronal Aβ in AD mouse models are quite consistent; intraneuronal Aβ was first reported in familial PS1 transgenic mice that also showed neurodegeneration but no plaques [15]. During the last years, it has been convincingly reported in several mouse models including $APP_{SDL}PS1_{M146L}$ [69], $APP_{SL}PS1_{M146L}$ [70], Tg2576 [60], 3xTg-AD [48], 5xFAD [47], APP_{Arc} [33, 40], APP_{T714I} mice [63], and in $APP_{SL}PS1KI_{M233T,L235P}$ mice [10] in which it was recently shown to correlate with neuron loss [8, 12, 14], as well as in a recently described mouse model expressing only pyroglutamate modified Aβ peptides within neurons [67].

Compared to mouse tissue, human tissue is more variable and factors like post-mortem delay, agonal state, and uneven tissue fixation, often of a prolonged duration, can interfere with immunohistochemical results. Although pH-dependent heat pretreatment is commonly used to reliably retrieve masked antigens from formalin-fixed, paraffin-embedded postmortem brain [41, 42, 58], most of the staining protocols for intracellular Aβ applied so far show considerable variation in use, duration and concentration of heat and formic acid (FA) treatment. Microwave heat pretreatment enhances the immunoreactive signal of intraneuronal Aβ as compared to conditions in which no or enzymatic pretreatment was applied [18]. FA is widely used to increase the staining of plaque pathology in AD; yet, the effect of FA on intraneuronal Aβ staining has been reported to be low and similar to the effect of heat [20], or even to counteract the enhancing effect of heat pretreatment on intraneuronal Aβ immunohistochemical detection [49]. On the other hand, formic acid pretreatment has been successfully used to improve aggregated α-synuclein staining in Lewy bodies and dystrophic neurites [61].

The present study optimized the staining protocol for intraneuronal Aβ using a highly specific N-terminal Aβ antibody on human AD tissue after testing various pretreatment conditions of heat and/or FA. The optimized protocol was then used to screen a series of sporadic AD cases and non-demented controls for the intensity of intraneuronal Aβ staining, which was then analyzed in a correlation analysis with patient data including ApoE genotype. A significant correlation between intraneuronal Aβ accumulation and the presence of at least one ApoE4 allele was established.

Materials and methods

AD brain tissue

Paraffin-embedded blocks from cortex and hippocampus of AD ($n = 20$) and control patients ($n = 10$) were acquired from the Netherlands Brain Bank (NBB, Amsterdam, The Netherlands) [51] that works with a rapid autopsy program and aims to keep postmortem delay (PMD) to a minimum. The NBB abides to all local ethical legislation, and permission was obtained for all brain autopsies and for the use of the tissues and clinical data for research purposes. To minimize variation as much as possible, control subjects were matched to the AD cases for age, sex, fixation time, pH of the cerebrospinal fluid, and PMD. Tissue of all subjects was investigated by a team of trained neuropathologists using a range of conventional and neuropathological stainings. The presence or absence of neuropathological changes was confirmed in the hippocampus and a range of other brain regions including the frontal and temporal cortex and the cerebellum. None of the control subjects had suffered from any primary neurological or psychiatric disease or brain metastases nor did they have a history of drug treatment or medication.

At autopsy, the hippocampus was dissected and fixed in 0.1 mol/L phosphate-buffered 4% formaldehyde (Sigma, St. Louis, MO, USA) solution (pH 7.2) for a period of approximately 4–5 weeks. Tissue was then dehydrated in graded ethanol and embedded in paraffin. 4-μm-thick tissue sections were cut for all patients from the midlevel of the hippocampus on a Leica microtome and mounted on

SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany). The following data were available for each patient: diagnosis, gender, age, post-mortem delay, disease duration, pH CSF, Braak stage, ApoE genotype, brain weight, as well as the medical history. In addition, information on extracellular plaque load was provided which contains a semi-quantitative score based on silver and 6F/3D (DAKO, Denmark, 1:400) immunostainings ranging from O (very few) to C (heavy plaque load) (Table 1).

The APOE genotyping of all patients was done according to standard protocols [66]. In brief, frozen human cortical samples were physically homogenized and powdered. DNA was then extracted and purified using standard methodology. Subsequently, parts of the APOE gene were amplified by means of PCR before the amplified DNA was fragmented using the restriction enzyme *Cfo*I that is single nucleotide polymorphism (SNP) sensitive. Fragment size was then evaluated from their mobility in PolyNat gel system stained with SYBR-Green [31].

Immunohistochemistry on formalin-fixed, paraffin-embedded sections

Immunohistochemistry was performed as described previously [70]. In brief, sections were deparaffinized in xylene and rehydrated in a series of ethanol. After treatment with 0.3% H_2O_2 in PBS to block endogenous peroxidases, either no further treatment was applied or antigen retrieval was performed by boiling sections for 10 min in a microwave oven in 0.01 M citrate buffer pH 6.0 and/or incubating sections for 3 min in 88% FA. After long-term fixation, various antigens can be masked by formalin crosslinks

Table 1 Clinicopathological data of the human brain material

Diagnosis	Gender	Age (years)	PMD (h:min)	Brain weight (g)	Braak stage	ApoE	Plaque load	Intraneuronal Aβ
AD	F	88	05:25	1,109	IV	ε4/ε3	C	+++
AD	M	91	04:10	1,160	IV	ε4/ε2	C	+++
AD	F	92	03:50	1,043	IV	ε4/ε2	C	+++
AD	M	81	04:50	1,253	IV	ND	C	+++
Control	M	78	18:00	1,222	I	ε4/ε3	C	+++
Control	F	90	07:15	1,047	I	ε2/ε2	B	+++
AD	F	91	03:45	1,011	IV	ε4/ε3	C	++
AD	F	84	10:00	1,206	IV	ε4/ε3	B	++
AD	F	79	05:20	1,213	IV	ε4/ε3	B	++
AD	F	91	04:15	1,202	IV	ε4/ε3	B	++
AD	F	86	03:45	1,098	IV	ε4/ε3	C	++
AD	M	92	03:30	1,175	IV	ε3/ε3	C	++
AD	F	88	03:30	1,002	IV	ε3/ε3	C	++
AD	F	88	12:15	935	IV	ε3/ε3	C	++
AD	F	86	04:10	1,083	IV	ε3/ε3	B	++
Control	M	70	07:45	1,560	0	ε4/ε3	B	++
Control	M	73	24:45	1,267	0	ε3/ε3	O	++
AD	F	87	02:55	928	IV	ε4/ε3	C	+
AD	M	93	05:50	1,220	IV	ε3/ε3	C	+
AD	F	92	05:10	1,110	IV	ε3/ε3	C	+
AD	F	93	06:15	943	IV	ε3/ε3	C	+
AD	F	84	04:15	1,023	IV	ε3/ε2	B	+
AD	F	85	02:45	1,247	IV	ε2/ε2	C	+
Control	M	84	09:00	1,367	I	ε3/ε3	O	+
Control	F	78	04:50	1,250	I	ε3/ε3	A	+
Control	F	82	11:30	1,159	I	ε3/ε3	O	+
Control	M	70	07:30	1,280	0	ε3/ε2	O	+
AD	M	86	06:00	1,377	IV	ε3/ε3	B	−
Control	M	91	<39:20	1,185	I	ε3/ε3	O	−
Control	F	88	03:45	1,195	I	ε3/ε3	O	−

Listed are diagnosis, sex, age, post-mortem delay (PMD, h:min), brain weight (g), Braak stage, ApoE genotype (combination of alleles 2, 3, and 4), extracellular plaque load, and intraneuronal $A\beta_{1-x}$ staining intensity

which hamper immunocytochemical detection. For many antigens, this can be overcome by heat-induced antigen retrieval [42, 57, 62]. Non-specific binding sites were blocked by treatment with 4% skim milk and 10% fetal calf serum in PBS, prior to addition of the primary antibodies. Primary antibodies used were directed against N-terminal Aβ peptides [Aβ[N], IBL (rabbit polyclonal, catalog No. 18584), Hamburg, Germany, 1:200] [35], G2-10 (Aβ40, The Genetics Company, Switzerland, 1:500), fibrillar Aβ oligomers (OC, generous gift of C. Glabe and R. Kayed 1:200) [32], $Aβ_{17–24}$ (4G8, Covance, 1:1,000), APP C-terminal (Synaptic Systems, Germany, 1:500) and glial fibrillary acidic protein (GFAP, Synaptic Systems, Germany, 1:1,000). All primary antibodies were incubated overnight in a humid chamber at room temperature. Single staining was visualized using the ABC method with Vectastain Kit (Vector Laboratories, Burlingame, USA) and 0.5 mg/mL DAB as chromogen providing a reddish brown color (10 min exposure for human tissue). In order to rule out a cross-reactivity of Aβ[N] with APP C-terminal fragments, SPA4CT mice overexpressing the β-cleaved APP C-terminal fragment under the control of the prion protein promoter [52], were stained with Aβ[N] and a C-terminal APP antibody (Supplementary Fig. 1).

Intraneuronal Aβ intensity was rated in the hippocampal region based on Aβ[N] staining intensity in CA4, CA3, and CA1 corresponding to either: no staining in any of the regions: 0 (−); weak staining in CA4 and CA3, but nothing in CA1: 1 (+); moderate staining in CA4 and CA3, but nothing or little in CA1: 2 (++); intense staining in all three regions: 3 (+++) (Fig. 4a–l).

Double immunocytochemical staining was visualized using the ABC method with DAB together with the HistoGreen kit (Linaris, Germany) providing a blue color. Counterstaining was carried out with hematoxylin.

Statistical analysis

Kolmogorov–Smirnov test was applied to examine which variables showed significant deviations from normal distribution. Most of the parameters, especially intraneuronal Aβ, showed significant deviation from normal distribution, and thus non-parametric tests were performed in the following analyses. Spearman rank correlations were calculated between intraneuronal Aβ and age, brain weight, extracellular plaque load, and post-mortem delay. Non-parametric Mann–Whitney *U* test was performed between intraneuronal Aβ and the factors gender, diagnosis, and number of ApoE 4 alleles. Non-parametric Kruskal–Wallis tests were computed between intraneuronal Aβ and Braak stage, where subgroup analysis between two groups was performed with Mann–Whitney *U* tests. As the study is explorative, the *P* values are given without Bonferroni adjustments. A binary logistic regression with dependent variable number of ApoE 4 alleles (0, ≥1) and independent variables intraneuronal Aβ, diagnosis, gender, age, and Braak stage was computed with the enter method, including all specified independent variables in the model. That way, the analysis on a correlation between intraneuronal Aß and ApoE 4 alleles was controlled for the indicated variables.

Results

Effect of formic acid and heat on staining of intraneuronal Aβ in AD patients

Optimization for intraneuronal Aβ staining was performed in hippocampal paraffin sections of sporadic AD cases using the Aβ N-terminal antibody detecting Aβ peptides starting with an aspartate at position 1. This antibody has been previously reported not to cross-react with full-length APP or β-C-terminal fragments [13, 35]. In addition, no cross-reactivity to APP C-terminal fragments could be demonstrated by staining SPA4CT mice overexpressing the β-cleaved APP C-terminal fragment C99. While strong immunoreactivity using a C-terminal APP antibody could be demonstrated in dentate gyrus granule cells and axonal fiber tracts like the corpus callosum, staining with the Aβ[N] antibody was consistently negative (Supplementary Fig. 1). Even without any antigen retrieval, a faint fairly homogenous intraneuronal $Aβ_{1-x}$ staining could be detected (Fig. 1a). Yet, 10-min heat treatment in 0.01 M citric acid buffer pH 6 dramatically increased the intraneuronal $Aβ_{1-x}$ staining showing a granular staining pattern and concentration around the nucleus (Fig. 1b). Compared to no pretreatment, 3-min FA pretreatment did not improve the staining of intracellular $Aβ_{1-x}$ (Fig. 1c), and actually very clearly counteracted the enhancing effect of the heat pretreatment on intraneuronal $Aβ_{1-x}$ staining (Fig. 1d). In addition to the fairly homogenous and punctate staining in the cytoplasm, some nuclei were observed to be surrounded by a highly granular Aβ staining pattern and were present in all three AD cases, and with all applied protocols (Fig. 1c). Double labeling for $Aβ_{1-x}$ and astrocytes using the Aβ[N] antibody visualized by DAB and the GFAP antibody visualized by Histogreen revealed that this highly abundant granular staining pattern in CA4 and CA3 of the hippocampal formation of many sporadic AD cases to be astrocytes accumulating Aβ (Fig. 2a, b, black arrows). In a few cases, astrocytes with no granular Aβ accumulation could be found in close proximity to neurons (Fig. 2c, d).

The presence of intraneuronal Aβ in the hippocampal region of sporadic AD cases was confirmed qualitatively by staining using the OC antibody recognizing Aβ fibrils and

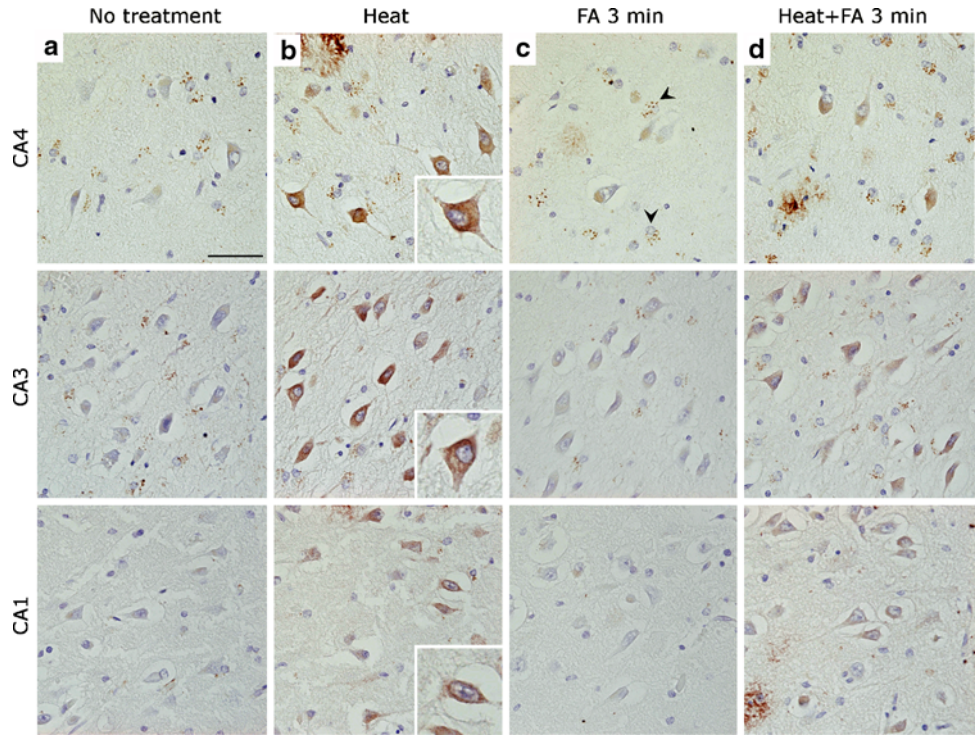

Fig. 1 Optimization for intraneuronal $A\beta_{1-x}$ staining in the hippocampal formation of sporadic AD cases using the Aβ[N] antibody in paraffin-embedded sections. A faint homogenous intraneuronal $A\beta_{1-x}$ staining could be detected even without any antigen retrieval (**a**). However, 10-min heat pretreatment in citric acid buffer pH 6 dramatically increased the intraneuronal $A\beta_{1-x}$ staining that at higher magnification showed granularity and concentration around the nucleus (**b**). Compared to no treatment, 3-min formic acid (FA) pretreatment failed to improve intraneuronal $A\beta_{1-x}$ staining (**c**). In the combined treatment of heat and FA, FA actually counteracted the enhancing effect of the heat pretreatment (**d**). Besides the intraneuronal $A\beta_{1-x}$ staining, smaller nuclei surrounded by a highly granular Aβ staining pattern were observed with all the protocols (**c**, *arrowheads*). *Scale bar* 50 μm

fibrillar oligomers [32]. The OC antibody produced an intraneuronal staining much like that of Aβ[N] with heat pretreatment alone (Fig. 3a, b) as well as with combined heat and 3-min FA pretreatment. The latter was actually slightly more intense than that enhanced by heat alone (Fig. 3c, d).

The widely used 4G8 antibody was applied to further confirm the presence of intraneuronal Aβ using heat pretreatment and was found to produce a prominent and intense granular staining. This pattern differed from that detected by OC or Aβ[N] antibodies (Fig. 3e, f), with the granules being much larger and surrounding the nucleus in a cap-like manner.

Intraneuronal Aβ staining in sporadic AD and control patients

Hippocampal sections from 10 controls and 20 sporadic AD patients were stained with the Aβ[N] antibody, and their intraneuronal $A\beta_{1-x}$ intensity was analyzed based on evaluation of the staining intensity in CA4, CA3, and CA1 (Fig. 4a–l). Four AD patients and two controls were found to accumulate the highest degree of intraneuronal $A\beta_{1-x}$ peptides, whereas nine AD patients and two controls accumulated a moderate amount of these Aβ peptides. Six AD patients and four controls accumulated low amount of Aβ peptides, whereas only one AD and two control patients were devoid of accumulation of $A\beta_{1-x}$ peptides (Table 1). By non-parametric statistical analysis, the accumulation of intraneuronal $A\beta_{1-x}$ was found to show no correlation with age, brain weight, post-mortem delay, gender, diagnosis, or Braak stage; however, a correlation between intraneuronal $A\beta_{1-x}$ accumulation and extracellular plaque load was established ($P = 0.0138$, $r = 0.4446$). Surprisingly, the ApoE genotype was found to strongly correlate with the presence of intraneuronal Aβ, where having one ApoE4 allele strongly correlated with increased intraneuronal $A\beta_{1-x}$ staining (Table 2; $P = 0.002$), which was also significant after Bonferroni adjustment ($P = 0.023$). Furthermore, from binary logistic regression with dependent variable ApoE 4 alleles and independent variables intraneuronal Aβ, diagnosis, gender, age, and Braak stage, there was only a significant influence of ApoE 4 alleles on intraneuronal Aβ (Wald statistic = 5.85; $P = 0.016$; odds-ratio = 16.38; 95% confidence interval = [1.70; 157.9]), while there was no significant influence of the other

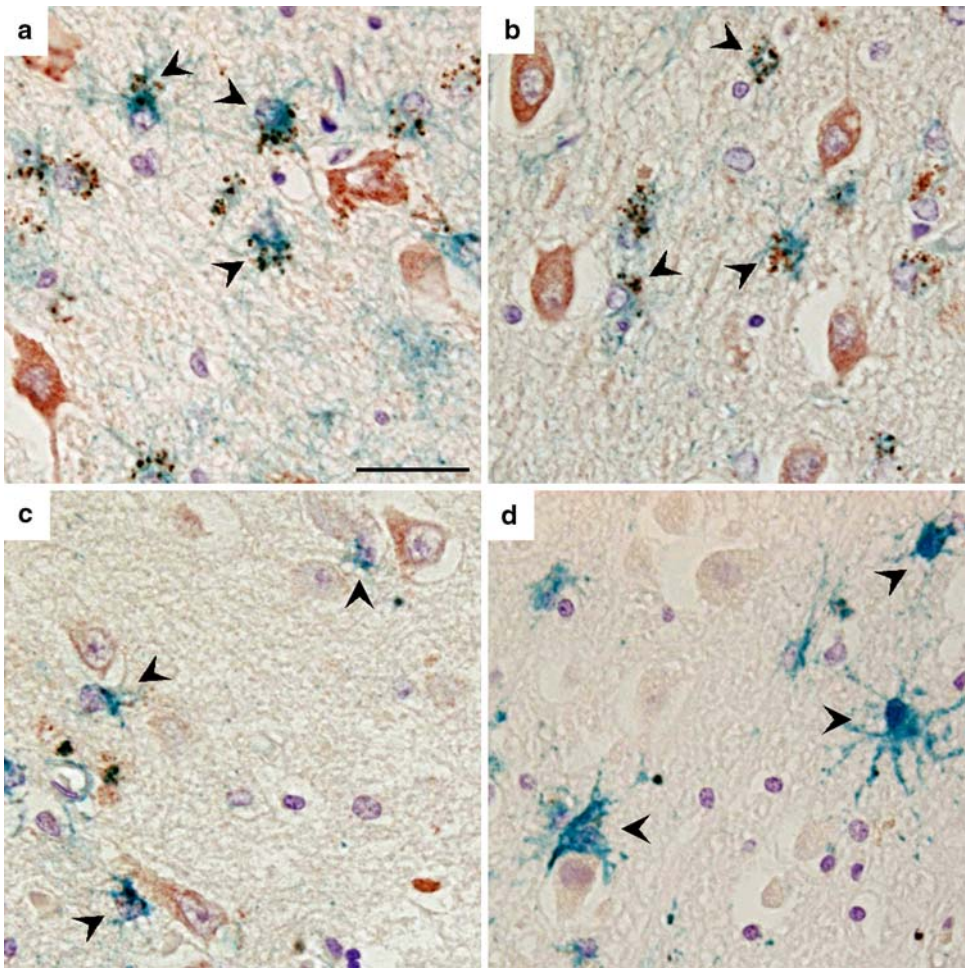

Fig. 2 Double labeling of $A\beta_{1-x}$ in *reddish brown* (DAB) using Aβ[N] antibody and astrocytes in *blue* (Histogreen, *black arrowheads*) using a GFAP antibody in paraffin-embedded sections. The highly granular staining pattern surrounding the smaller nuclei was due to astrocytes accumulating Aβ and was found in CA4 (**a**) as well as in CA3 (**b**) of many sporadic AD cases. However, in some AD cases, astrocytes without granular staining were found close to neurons in both CA4 (**c**) and CA3 (**d**). *Scale bar* 33 μm

independent variables that the analysis was adjusted for. The rate of correct classifications for ApoE 4 alleles increased from 62 to 79% after the independent variables were entered to the model.

To confirm the results of the Aβ[N] staining in the detection of intraneuronal Aβ peptides in human post-mortem tissue, the same cohort of patients was stained using the commercially available antibody G2-10, which recognizes a neo-epitope at the C-terminus of Aβ40 peptides (Supplementary Fig. 2). A correlation analysis between the staining pattern of the two antibodies revealed a highly significant correlation ($P = 0.002$; $r = 0.55$), corroborating the validity of the staining protocol.

Discussion

The effect of heat and FA pretreatment was semi-quantitatively investigated in human AD tissue using a highly specific N-terminal-specific antibody detecting $A\beta_{1-x}$. It has been previously reported that this antibody specifically recognizes Aβ peptides, without cross-reaction to APP or APP C-terminal fragments [13, 35]. The optimization was performed in paraffin-embedded hippocampal sections from sporadic AD cases by means of combinations of acid and heat pretreatments, essential conditions for antigen retrieval from brain tissue fixed for prolonged periods of time [42, 57, 62]. As the Aβ[N] antibody is directed against the aspartate at position 1 of Aβ, it might also detect APP C-terminal fragments bearing the same epitope. To overcome this problem, we stained tissue sections from a transgenic mouse model overexpressing the β-cleaved APP C-terminal fragment C99 (SPA4CT mice) [52], and found no evidence for any cross-reactivity to β-CTF in formalin-fixed and paraffin-embedded material. However, residual cross-reactivity using other techniques cannot be completely ruled out. Some intraneuronal $A\beta_{1-x}$ staining was evident even without any pretreatment, but heat dramatically increased the intraneuronal staining of smaller granules throughout the cytoplasm concentrating around the nucleus in all investigated hippocampal regions, especially in the CA4 region. In contrast, FA pretreatment did not increase the staining of intraneuronal $A\beta_{1-x}$ as compared to sections

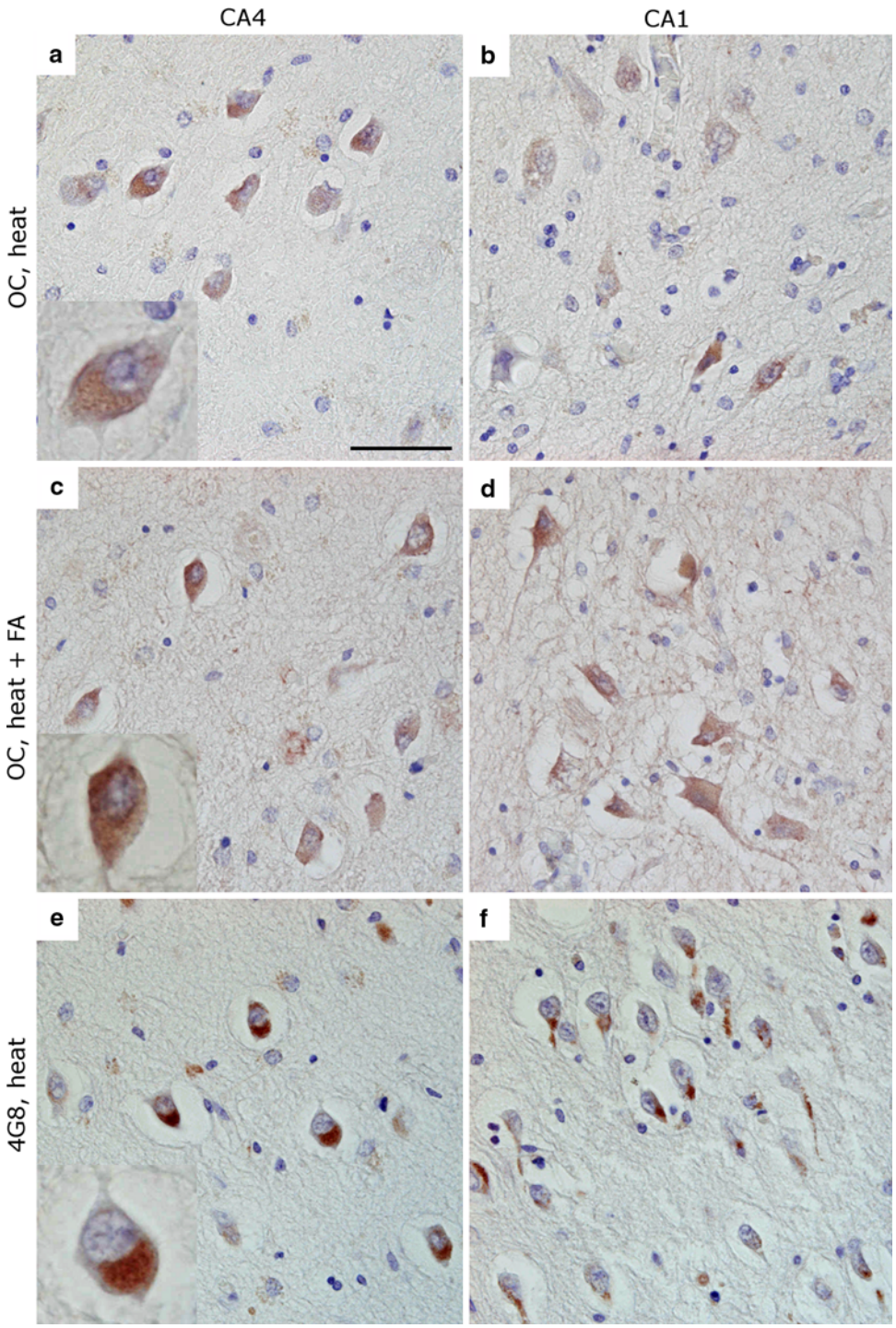

Fig. 3 Intraneuronal staining detected by OC and 4G8 antibodies in a sporadic AD brain. The OC antibody disclosed intraneuronal Aβ staining much like that of Aβ[N] with both 10-min heat pretreatment alone (**a**, **b**) and combined heat and 3-min formic acid (FA) treatment (**c**, **d**). With 10-min heat pretreatment, the 4G8 antibody produced a different, highly abundant and granular intracellular staining (**e**, **f**). *Bottom left corners* of **a**, **c**, and **e** show intracellular staining at a high magnification. *Scale bar* 50 μm

where no antigen retrieval was applied. In fact, FA even reduced the enhancing effect of heat; the combination of heat and FA only slightly increased the staining of $A\beta_{1-x}$ as compared to no pretreatment (Fig. 1d, h, l), resulting in much less retrieval than heat alone. The counteracting effect of FA on the heat-induced staining of intraneuronal $A\beta_{1-x}$ peptides corroborates findings of another study using an Aβ42(43) specific antibody (BC-05) and an autoclave heating protocol that reported a counteracting effect of FA on heat-induced Aβ42 staining [49] and is in line with previous studies showing that FA preferentially retrieves nuclear rather than cytoplasmic antigens [7, 57, 62]. Taken together, the strong improvement in intraneuronal Aβ immunoreactive signal after this pretreatment

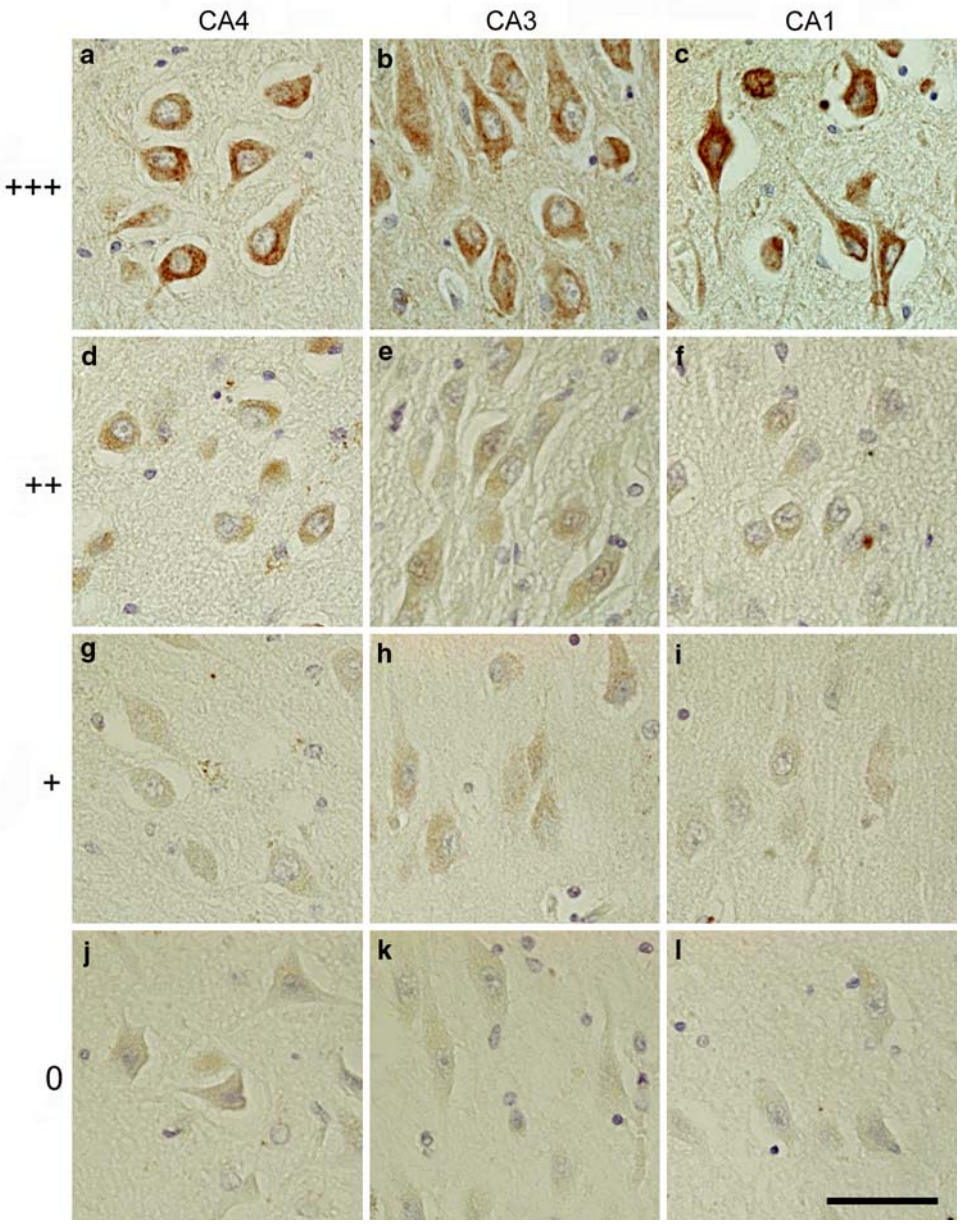

Fig. 4 Rating of intraneuronal $A\beta_{1-x}$ staining intensity. +++ was assigned to cases with very strong intraneuronal Aβ staining in CA4, CA3, as well as in CA1 (**a**–**c**). ++ was assigned to cases with weaker but still obvious intracellular Aβ staining in CA4 and CA3 and low staining in CA1 (**d**–**f**). + was assigned to cases with a very faint intracellular Aβ staining in CA4 and CA3 and apparently no staining in CA1 (**g**–**i**). 0 was assigned to cases showing no intracellular Aβ staining either in CA4, CA3, or CA1 (**j**–**l**). *Scale bar* 50 μm

may explain discrepancies with older literature, where antigen retrieval was generally not applied.

These findings are in contrast to the observations in mouse models, where FA clearly enhanced staining of intraneuronal Aβ peptides with heat yielding no, or only a minor increase [13]. The difference in the effect of heat and FA between mouse and human tissue could be explained by differences in the Aβ species that accumulate in the cells. In the AD mouse models, much of the intraneuronal Aβ induced by FA pretreatment seemed to be aggregated peptides. Possibly, the intraneuronal $A\beta_{1-x}$ in AD tissue could reflect soluble oligomeric species that may be enhanced by heat but counteracted by FA pretreatment. In agreement, low-*n* oligomeric Aβ have been detected inside primary human neurons [64].

The presence of intraneuronal Aβ in sporadic AD tissue was further supported by staining with the polyclonal OC antibody recognizing fibrillar Aβ oligomers and Aβ fibrils [32]. An intense intraneuronal Aβ staining was observed after heat pretreatment as well as with additional FA pretreatment that enhanced intraneuronal OC staining. This is in contrast to the deleterious effects of FA pretreatment on the detection of $A\beta_{1-x}$, but agrees with the suggestion that FA enhances the staining of aggregated Aβ fibrils recognized by the OC antibody. The staining retrieved by heat pretreatment could thus be due to detection of oligomeric

Table 2 Spearman correlation between intraneuronal Aβ and the dichotomic dependent variables sex, diagnosis, and the factor ApoE4 alleles by non-parametric Mann–Whitney *U* tests

	Intraneuronal Abeta
Gender	
Male	
N	11
M	1.64
SD	1.12
Female	
N	19
M	1.68
SD	0.82
Z	−0.1
df	1
P	0.95
Diagnosis	
Control	
N	10
M	1.40
SD	1.08
AD	
N	20
M	1.80
SD	0.83
Z	−1.1
df	1
P	0.31
ApoE4	
No ApoE4 alleles	
N	18
M	1.22
SD	0.81
1 ApoE4 allele	
N	11
M	2.27
SD	0.65
Z	−3.1
df	1
P	0.002

Aβ fibrils by the OC antibody, which is consistent with two previous studies suggesting that Aβ oligomerization starts within neurons [59, 64].

It is known that besides Aβ peptides starting with an aspartate at position 1, a variety of different N-terminal truncated Aβ peptides have been identified in AD brain tissue [28, 53]. Especially compact Aβ deposits in human brain often contain pronounced N-terminal degradation and post-translational modifications [36]. While N-terminal modifications of Aβ have been identified to occur within neurons [8], it is likely that the majority of intraneuronal Aβ peptides start with the aspartic acid at position 1 as cleavage by BACE is the initial step for Aβ generation.

Intraneuronal Aβ originates exclusively from intraneuronal sources. Whether it can, in addition, be internalized from external sources is not yet clarified. Yet, much evidence supports the possibility of a reuptake of Aβ peptides into cells by endocytosis, and members of the lipoprotein receptor (LDLR) family [9], α7 nicotinic receptors [46], as well as scavenger receptor for advanced glycation end products (RAGE) [21, 56, 71] have all been reported to interact with Aβ and to be capable of internalization of extracellular Aβ peptides. In particular, RAGE-Aβ complexes have been shown to be internalized and co-localize with the lysosomal pathway in astrocytes in AD brains [56]. This is supported by our observation of astrocytes accumulating high amounts of Aβ granules.

The LDLR-related protein (LRP) is a member of the LDLR family and functions as an ApoE receptor. LRP has been shown to facilitate rapid endocytosis of APP promoting APP processing and thus Aβ generation. In addition to the effect on APP trafficking, LRP-induced rapid endocytosis also facilitates cellular uptake of Aβ peptides from the extracellular space, either directly through binding to Aβ or indirectly through interaction with ligands such as ApoE [9]. Accordingly, knock out of APOE in PDAPP transgenic mice reduced the accumulation of intracellular Aβ [72]. Given this possible role for ApoE in the intraneuronal Aβ accumulation, we correlated the intensity of the intraneuronal $A\beta_{1-x}$ staining in the hippocampal regions of 20 AD patients and 10 controls to ApoE genotype and found a highly significant correlation between having one ApoE4 allele and the intensity of $A\beta_{1-x}$ staining ($P = 0.002$). This is an interesting finding as ApoE4 plays an important role in neurodegeneration in general and in AD in particular (reviewed in [43]). The ε4 allele of the *ApoE* gene is the major known genetic risk factor for AD with a frequency of ∼15% in general populations but >50% in AD patients [16]. Moreover, ApoE4 influences beta-amyloid degradation [73] brain and neuronal activity [39, 55] while senile plaques are more frequent in ApoE4-carriers compared to non-carriers. This was most evident during the age of 50–59 years, with ∼41% of ε4-carriers bearing senile plaques, compared to only ∼8% in non-carriers [34]. This suggests that there is a differential development of AD-associated changes in the brain of individuals having at least one ε4 allele, which starts already in middle age.

Crossing PDAPP mice with mice in which the endogenous mouse ApoE was substituted with human ApoE2, ApoE3 or ApoE4 resulted in a substantial and early increase in brain Aβ42 levels, prior to extracellular plaque deposition [5]. In addition, intraneuronal Aβ accumulation

has been previously linked to ApoE. PDAPP mice overexpressing mLRP2 had higher hippocampal detergent-soluble Aβ42 levels than PDAPP wildtype mice. Furthermore, cortical intraneuronal Aβ42 was significantly reduced in PDAPP mice lacking ApoE, leading to the assumption that ApoE facilitates intraneuronal Aβ accumulation [72].

ApoE4-transgenic mice housed in an enriched environment showed increased levels of oligomerization and deposition of Aβ peptides in hippocampal neurons compared to ApoE3-transgenic mice housed under the same conditions [38]. Furthermore, inhibition of the Aβ-degrading enzyme neprilysin in ApoE3 and ApoE4 mice results in an ApoE4 isoform-specific degeneration of hippocampal CA1 neurons, which is accompanied by intracellular accumulation of Aβ and ApoE and lysosomal activation [6]. In good agreement with this lysosomal pathology, altered endocytic pathway activity is one of the earliest known intraneuronal changes occurring in sporadic AD, and it has been shown that the ApoE4 genotype promotes early-stage endocytic pathway activation [11]. In conclusion, having optimized techniques for reliable detection of intraneuronal Aβ, our subsequent analysis revealed a strong association between the ApoE4 genotype and the presence of intraneuronal Aβ. These results confirm recent data obtained in experimental settings and are consistent with an important role for intracellular Aβ metabolism, possibly mediated through ApoE4, in AD etiology.

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