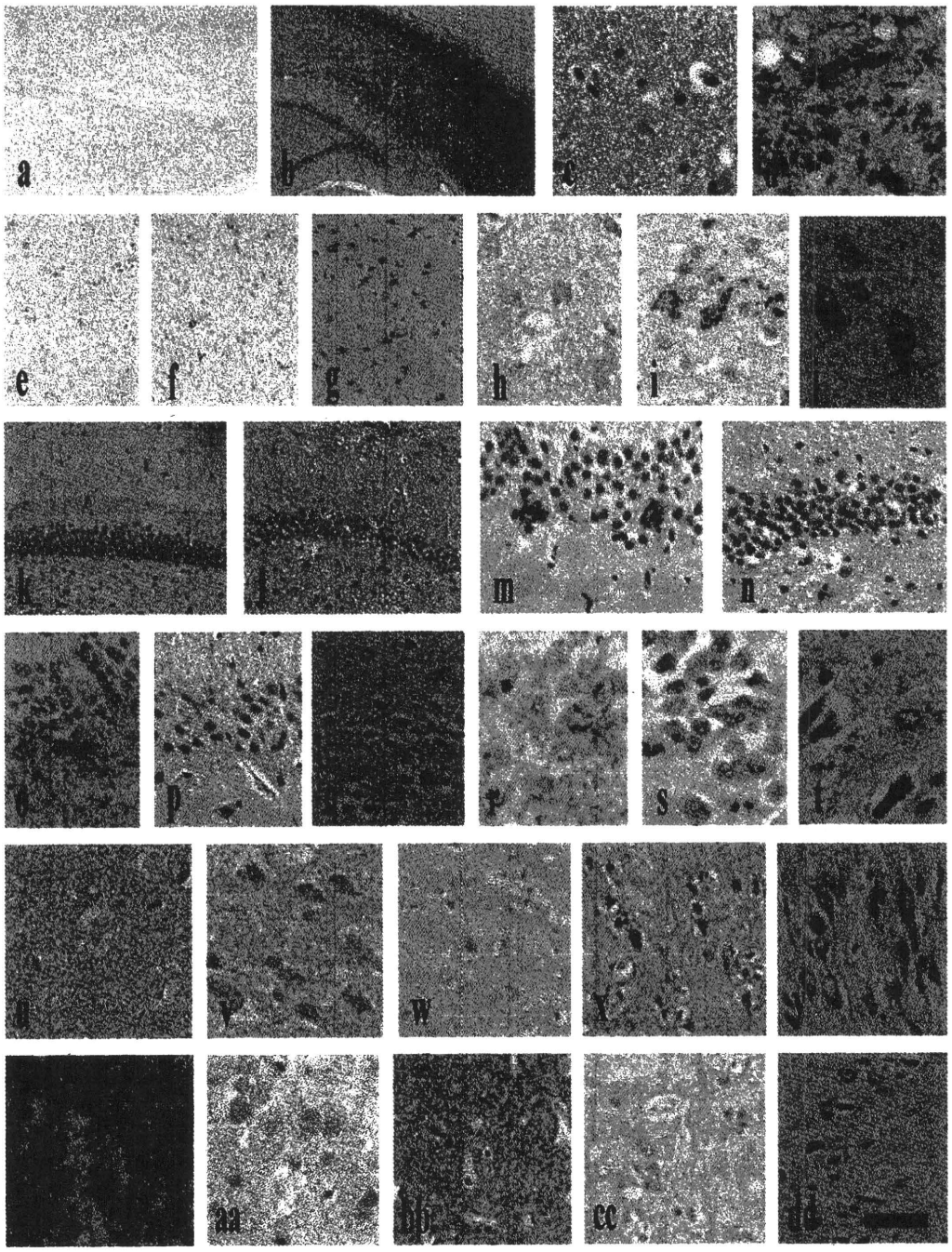

Fig. 2. Immunostaining of ApoD, DCX, Npas4, and Kcnab1. Staining of ApoD (from Patel; **a–d**), ApoD(36C6) (**e–j**), DCX (N19; **k–t**), Npas4 (polyclonal; **u–y**), and Kcnab1 (**z–dd**) in TgTauP301L without NFTs and neuronal cell losses (a,c,k,m,u,z), with NFTs and neuronal cell losses (b,d,l,n,v,aa), control human brains (e,h,o,r,w,bb), AD brains (f,i,p,s,x,cc), and FTD brains (g,j,q,t,y,dd). Staining of ApoD and Npas4 was increased in TgTauP301L brain with NFTs and neuronal cell losses, AD brains, and FTD brains compared with that in TgTauP301L brain without NFTs and neuronal cell losses and control brain. Decreased immunoreactivities of DCX and Kcnab1 were recognized in TgTauP301L brain with NFTs and neuronal cell losses, AD brain, and FTD brains. Scale bar = 50 μm in dd (applies to e–g,m–q,w–y,bb–dd); 250 μm for a,b; 100 μm for c,d,k,l; 25 μm for h–j,r–v,z,aa. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

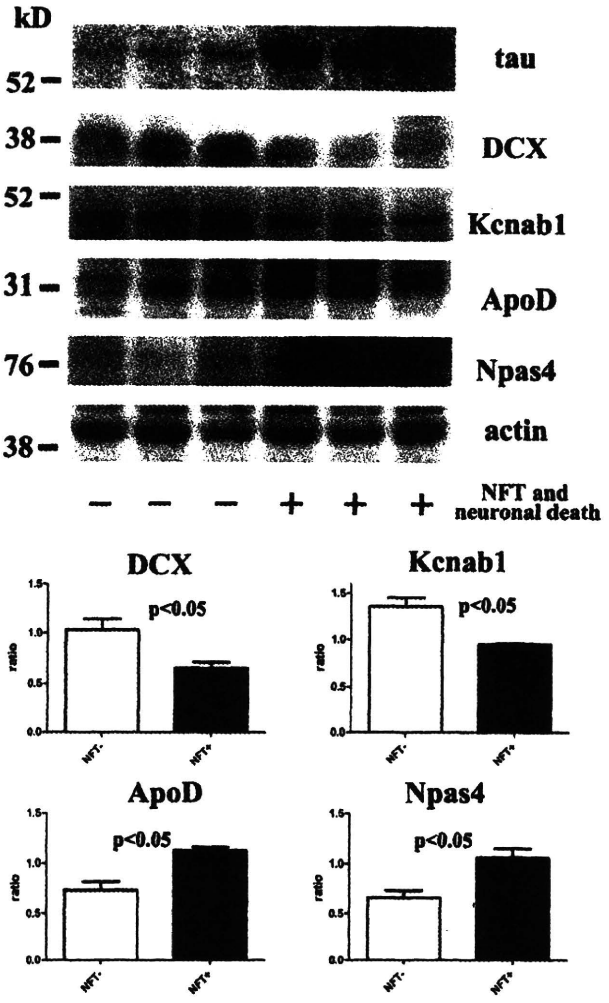

Fig. 3. Western blotting of brain extracts using tau (TAU-5), DCX (C-18), Kcnab1, ApoD, and Npas4 antibodies. Insoluble tau accumulation (TAU-5) was correlated with decreased levels of DCX (C18) and Kcnab1 and increased levels of ApoD (from Patel) and Npas4 (NBP1-06574). Plus signs indicate brain extracts of TgTauP301L with NFTs and neuronal cell losses, and minus signs indicate those of TgTauP301L without NFTs and neuronal cell losses. The signals were corrected by those of β-actin and were tested for statistical significance by using *t*-test.

NFT formation and neuronal cell losses at the gene product level (Fig. 3).

Immunostaining of Npas4 was increased in cerebral cortex and hippocampus of TgTauP301L brains with NFTs and neuronal losses (Fig. 2v) and also in AD and tauopathy brains (Fig. 2x,y). Immunoreactivity of Kcnab1 was decreased in cortex and hippocampus of TgTauP301L brains with NFTs and neuronal losses (Fig. 2aa) and also in AD and tauopathy brains (Fig. 2cc,dd). On Western blotting, the amount of Npas4 was increased and the level of Kcnab1 was decreased in TgTauP301L mice with NFTs and neuronal cell losses compared with those in TgTauP301L without NFTs and neuronal cell losses (Fig. 3). Therefore, changed expression level of Npas4 and Kcnab1 was also verified at the protein level.

DISCUSSION

Our oligonucleotide array expression study demonstrated that gene expression groups were altered in many biological pathways and that these collective alterations were closely related to the final stage of tauopathy, namely, NFT formation and neuronal cell losses. Both sets of comparisons of mice with or without NFTs and neuronal cell losses showed that five pathways including oxidative stress, apoptosis, mitochondrial fatty acid betaoxidation, inflammatory response, and complement and coagulation cascades are consistently up-regulated, suggesting that NFTs and neuronal losses occurred on the basis of at least these enhanced biological pathways with many up-regulated gene cluster expressions. Then, altered gene expression ratios demonstrated in both comparison sets consisting of 17 up-regulated genes and seven down-regulated genes and biological processes were summarized by Gene Ontology (Table I). Reported roles of up-regulated genes were classified into inhibitory synapse development (Npas4), metabolism (thyrotropin-releasing hormone, insulin receptor substrate 4, pyruvate dehydrogenase kinase, isoenzyme 4), inflammation [immunoglobulin heavy chain 6, C-type lectin domain family 7, member a, serine (or cysteine) peptidase inhibitor, clade A, member 3N, carboxypeptidase M], brain development (syndecan 4), CNS myelination (predicted gene 98, myelin-associated oligodendrocytic basic protein), cell cycle regulation (Holliday junction recognition protein), protein kinase or phosphatase activity (calcium/calmodulin-dependent protein kinase II inhibitor 1), body weight regulation (perilipin 4), proteosome system [proteasome (prosome, macropain) 26S subunit, ATPase 3, interacting protein], lipid transportation (ApoD), and tumorigenesis (periostin, osteoblast specific factor). These findings indicated that NFT formation and neuronal cell losses were associated with up-regulated conditions of CNS regeneration, metabolism, lipid transportation, proteosome function, cell cycle regulation and signaling, and inflammation. Down-regulated genes were classified into photoreceptor synaptic calcium handling (membrane protein, palmitoylated 4), neuronal protection (pituitary tumor-transforming gene 1), neurogenesis (DXC), synaptic plasticity and learning (Kcnab1), mitochondrial function (succinate dehydrogenase complex, subunit A, flavoprotein), and others (RNA binding motif protein 45, RIKEN cDNA 4933400F03 gene). These conditions can also be summarized as suppressed neuronal and synaptic function, neurogenesis, and mitochondrial dysfunction.

Among these NFTs and neuronal cell loss-related gene expressions, ApoD has been reported to be elevated in the brains and cerebrospinal fluid of AD patients

(Terrisse et al., 1998; Kalman et al., 2000). ApoD is detected in oligodendrocytes, astrocytes, neurons with NFTs, and the vicinity of senile plaques, and the level of ApoD is associated with the severity of NFTs, but not that of Aβ (Belloir et al., 2001; Glöckner and Ohm, 2003). Accumulation of ApoD has been reported in another tauopathy, Nieman Pick type C brains (Ong et al., 2002). ApoD rapidly increases with various types of brain damage and peripheral nerve regeneration (Boyles et al., 1990) and is involved in the mechanisms regulating protection from oxidative stress (Andersen, 2004; Ganfornina et al., 2008). In our study, the level of ApoD was prominently increased in the brains of mice with NFTs and neuronal cell losses as well as gene expressions. Because our mice and Nieman Pick type C patients exhibit tauopathy findings without Aβ, the increased level of ApoD appears closely related to NFT formation and neuronal cell losses. NFT-mediated neurotoxicity and increased oxidative stress may increase the level of ApoD.

We also found that the expression of DCX gene and the amount of DCX were decreased in brains with NFTs and neuronal cell losses. The level of DCX is inversely correlated with accumulation of insoluble tau aggregation. DCX is one of the microtubule-binding proteins for axonal growth and collateral branching and essential in postmitotic neurons for brain development (Moores et al., 2004; Tint et al., 2009), indicating that DCX is a marker of neurogenesis. Although a first report suggested an increased amount of DCX accompanying hippocampal neurogenesis in AD brains (Jin et al., 2004), others did not show consistent results (Boekhoorn et al., 2006a; Verwer et al., 2007). Aβ decreases the DCX-positive cells in the hippocampus in APP model mice (Zhang et al., 2007; Mirochnic et al., 2009). In tau transgenic mouse models, however, DCX-positive neurogenesis was increased in an early phase (Boekhoorn et al., 2006b; Schindowski et al., 2008). In the late stage with NFT formation, up-regulation of cell cycle events and decreased DCX levels occurred, accompanied by neuronal cell losses (Schindowski et al., 2008). These findings corresponded to our findings and suggest that suppressed neurogenesis is one of the causal factors underlying neuronal cell losses in brains with tauopathy.

Npas4 is a newly identified transcription factor that plays a role in the development of inhibitory synapses (Lin et al., 2008) and stress-induced impairment of hippocampal function (Yun et al., 2010). Kcnab1 is also associated with excitability in hippocampal neurons and impairment of learning and memory (Need et al., 2003). Altered gene expressions of both hippocampal proteins were confirmed at protein levels. Although roles of Npas4 and Kcnb1 have not been precisely clarified, our findings suggest that these novel molecules participate in cascades from tau accumulation to NFT formation and neuronal cell losses leading to memory disturbance. Thus, gene expression profile and confirmations at protein level are useful methods for clarifying the constituent molecules of tauopathy.

Recent studies in an NFT mouse model showed that NFTs are neurotoxic and cause delayed neuronal cell losses without the usual caspase-dependent apoptosis leading to acute neuronal cell losses (Spires-Jones et al., 2008; de Calignon et al., 2009). Age-dependent reinforcement of tau accumulation and mitochondrial dysfunction-related energy metabolism and oxidative stress leads to NFTs and apoptotic neuronal cell losses (Kulic et al., 2010). Given these findings, neuronal cell losses may be caused by NFT neurotoxicity, suppressed neurogenesis, and apoptosis accompanying NFT formation induced by many biological pathways. To develop therapy for neuronal cell losses as a final target of tauopathy, every step of these biological pathways and key molecules must be clarified.

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ORIGINAL PAPER

Capillary cerebral amyloid angiopathy identifies a distinct *APOE* ε4-associated subtype of sporadic Alzheimer's disease

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Abstract The deposition of amyloid β-protein (Aβ) in the vessel wall, i.e., cerebral amyloid angiopathy (CAA), is associated with Alzheimer's disease (AD). Two types of CAA can be differentiated by the presence or absence of capillary Aβ-deposits. In addition, as in Alzheimer's disease, risk for capillary CAA is associated with the apolipoprotein E (*APOE*) ε4-allele. Because these morphological and genetic differences between the two types of AD-related CAA exist, the question arises as to whether there exist further differences between AD cases with and without capillary CAA and, if so, whether capillary CAA can be employed to distinguish and define specific subtypes of AD. To address this question, we studied AD and control cases both with and without capillary CAA to identify the following: (1) distinguishing neuropathological features; (2) alterations in perivascular protein expression; and (3) genotype-specific associations. More widespread Aβ-plaque pathology was observed in AD cases with capillary CAA than in those without. Expression of perivascular excitatory amino acid transporter 2 (EAAT-2/GLT-1) was reduced in cortical astrocytes of AD cases with capillary CAA in contrast to those lacking capillary Aβ-deposition and controls. Genetically, AD cases with capillary CAA were strongly associated with the *APOE* ε4 allele compared to those lacking capillary CAA and to controls. To further validate the existence of distinct types of AD we analyzed polymorphisms in additional apoE- and

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cholesterol-related candidate genes. Our results revealed an association between AD cases without capillary CAA (i.e., AD cases with CAA but lacking capillary CAA and AD cases without CAA) and the T-allele of the $\alpha_2$macroglobulin receptor/low-density lipoprotein receptor-related protein-1 (*LRP-1*) C766T polymorphism as opposed to AD cases with capillary CAA and non-AD controls. Taken together, these results indicate that AD cases with capillary CAA differ significantly from other AD cases both genetically and morphologically, thereby pointing to a specific capillary CAA-related and *APOE* ε4-associated subtype of AD.

Keywords Alzheimer's disease · Cerebral amyloid angiopathy · Apolipoprotein E (*APOE*) · α_2Macroglobulin receptor/low-density lipoprotein receptor-related protein (*LRP-1*) · EAAT-2

Introduction

Increasing evidence points to the hypothesis that amyloid β-protein (Aβ) aggregates are of major importance for the development of Alzheimer's disease (AD) [30, 41, 85]. However, the etiology of the disease is by no means fully understood. In addition to Aβ, a number of other factors have also been implicated in the pathogenesis of AD: abnormal τ-protein [5, 9, 29], oxidative stress [15], altered cholesterol metabolism [31, 56], neuroinflammation [26, 27, 46, 47], blood brain barrier (BBB)-related or perivascular clearance dysfunction [78, 83, 88], and mitochondrial alterations [21, 79]. The apolipoprotein E (*APOE*) ε4 allele is widely recognized as a risk factor for sporadic AD [8, 19, 62], whereas most other candidate genes remain elusive owing to subsequent replication failure [8]. Furthermore, based on their *APOE* ε4 carrier status, AD patients display pharmacogenetic differences [59]. To date, it is unclear whether sporadic AD is a single, monolithic disorder with varying degrees of severity or whether it comprises multiple subtypes that possibly bear a strong resemblance to each other but differ with respect to pathophysiology, disease course, and/or treatment response.

Aβ deposition in cortical capillaries is strongly associated with the *APOE* ε4-allele and permits differentiation between two types of cerebral amyloid angiopathy (CAA): capillary CAA (i.e., CAA-type 1), which is associated with the presence of the *APOE* ε4 allele, and CAA without capillary Aβ-deposition (i.e., CAA-type 2), which is not associated with the *APOE* ε4-allele [68]. CAA-type 1 was more frequently found in AD cases than CAA-type 2 [3, 69]. Capillary CAA follows a similar hierarchical sequence of expansion throughout the brain regions as CAA in arteries and veins whereby the basal ganglia, insular cortex, and the medulla oblongata are spared [69].

The goal of the present study was to determine whether capillary CAA constitutes a distinct AD subtype that differs both in Aβ and/or neurofibrillary pathology as well as in the integrity of the pericapillary neuropil from AD cases that lack capillary CAA. We examined a sample of 71 AD cases and 309 controls for the overall distribution of Aβ and neurofibrillary tangle pathology. Because the *APOE* ε4 allele constitutes a major risk factor for AD and CAA-type 1, we analyzed the association of the *APOE* ε4 allele in AD cases with and without capillary CAA. In addition, given that apoE is involved in cholesterol trafficking in the brain [80], we also analyzed the association between AD cases with and without capillary CAA and polymorphisms in cholesterol metabolism- and apoE-related candidate genes that have been reported previously to be associated with AD [40, 44, 45, 54, 55]. Moreover, capillary CAA contributes to capillary occlusion with the result that cerebral blood flow is impaired, usually without tissue necrosis [57, 66]. Likewise, aged *APOE* ε4-carriers develop a widespread deficiency in regional cerebral blood flow in comparison to non ε4-carriers [75], thereby reinforcing the hypothesis that *APOE* ε4-associated capillary CAA may lead to alterations in cerebral blood flow. It is not known whether capillary CAA and its adverse effects are associated with cellular alterations of cortical astroglia that constitute the glia limitans. Recently, however, Zhong et al. [87] reported reduced levels of the astroglial glutamate transporter EAAT-2 (excitatory amino acid transporter 2, synonymous with GLT-1/SLC1A2) expression in a mouse model for apoE4-like domain interactions. Thus, we decided to focus our attention also on the expression of the astroglial glutamate transporters EAAT-1 (excitatory amino acid transporter 1, synonymous with GLAST/SLC1A3) and EAAT-2 to investigate potential differences between perivascular astrocytes in cases with and without capillary CAA and, in so doing, to identify further differences between AD cases with and without capillary CAA. The occipital cortex was chosen to study the glutamate transporter expression in the presence or absence of capillary CAA because this region is a predilection site for capillary CAA [69].

Here, our results corroborate the hypothesis that the presence or absence of capillary CAA makes it possible to differentiate at least two types of sporadic AD supported by type-specific genetic associations and differences in the EAAT-2 expression in perivascular astrocytes.

Materials and methods

Neuropathology and human sample characterization

A sample of 380 non-selected autopsy cases aged ≥50 years was investigated. This sample included 71 AD and 309 control cases (comprising 259 non-demented and 50 demented non-AD cases, Tables 1, 2). All autopsy brains were collected from individuals, who died in university or municipal hospitals in the European Union (Bonn, Offenbach am Main, Ulm, Germany, Enschede, The Netherlands, and Vienna, Austria) and in the USA (Little Rock, AR, USA) and all were obtained with local legal and ethical committee approval. Demented as well as non-demented patients were examined 1–4 weeks prior to death using standardized protocols for routine examination of patients, including neurological status, upon admission to hospital. These protocols included assessment of cognitive function and the ability to perform activities of daily living: grooming and dressing oneself, meal preparation, bladder and bowel continence, speech patterns, reading and writing skills, short-term and long-term memory, and orientation within a hospital setting. The Clinical Dementia Rating (CDR) score [33] was observed retrospectively for 47 AD cases. These data were used to determine whether individuals clinically fulfilled the DSM-IV criteria for dementia [2]. In 24 AD cases, the clinical records noted the diagnosis of dementia according to DSM-IV. AD was diagnosed when dementia was observed and when the degree of AD-related neuropathology indicated at least a moderate likelihood for AD according to acknowledged criteria [76]. Causes of death were heterogeneous including anaphylactic reaction (0.3%), brain disorders (8.6%), cancer (15.5%), heart disease (28.2%), gastrointestinal disorders (2.2%), infectious diseases (including pneumonia, 17.7%), metabolic disorders (2%), respiratory failure (22%), vascular disorders (except coronary heart disease and cerebrovascular disorders, 2.5%), and trauma (1%). In the event that brain disorders other than AD were diagnosed, this information appears in Table 2. Pathological determination of the cause of death was not possible in 21 cases as only brain autopsy was permitted, and, therefore, were not included in the cause of death statistic.

The brains were fixed in a 4% aqueous formaldehyde solution for at least 3 weeks and underwent neuropathological screening. Presence or absence of gross infarction, hemorrhage, tumor, and other findings were recorded. Blocks from the medial temporal lobe (MTL) were excised at the levels of the (1) anterior limit of the dentate gyrus, (2) lateral geniculate body, and (3) posterior limit of the dentate gyrus [35]. All tissue blocks were embedded in polyethylene glycol (PEG, Merck–Schuchardt, Hohenbrunn, Germany) [11] and/or in paraffin. Tissue blocks from the occipital cortex (Brodmann areas 17, 18, 19) were embedded in paraffin. The PEG-blocks were sectioned on a sliding microtome at 100 μm, whereas the paraffin sections were sectioned at 10 μm.

Neurofibrillary changes were detected using the Gallyas silver-staining method and/or by immunostaining with an antibody directed against abnormal phosphorylated τ-protein (AT-8, s. immunohistochemistry) [10, 11, 36]. Neuritic plaques were diagnosed either in Gallyas- or Bielschowsky-stained sections or in sections immunostained with an antibody directed against hyperphosphorylated τ-protein (AT-8, s. immunohistochemistry). The presence of amyloid deposition was assessed using the Campbell–Switzer silver impregnation method [11, 36] and/or immunohistochemistry (4G8, s. immunohistochemistry). Both methods for the detection of amyloid plaques (including diffuse plaques and fleecy amyloid) produce nearly identical results and are interchangeable [72, 73]. CAA was studied in anti-Aβ immunostained sections.

Diagnosis of the stages in the development of neurofibrillary changes (Braak NFT-stage) and the semiquantitative assessment of neuritic plaques (CERAD-score) were performed in accordance with published and recommended criteria [10, 12, 49, 76]. For staging of Aβ-pathology, we used a previously published protocol for four phases of β-amyloidosis in the MTL [72]. This hierarchically based procedure facilitates study of the topographic distribution pattern of Aβ-deposition in additional brain regions [71, 72]: *Phase 1* represents Aβ-deposition that is restricted to the temporal neocortex. *Phase 2* is characterized by the presence of additional Aβ-plaques in the entorhinal cortex and/or in the subiculum-CA1 region. The *third phase* of β-amyloidosis is marked by the presence of Aβ-plaques in the outer zone of the molecular layer of the fascia dentata, subpial band-like amyloid, and/or presubicular "lake-like" amyloid. The existence of further Aβ-plaques in CA4 and/or the pre-α layer of the entorhinal cortex characterize the *fourth* and *final phase* of Aβ-deposition in the MTL.

CAA was diagnosed whenever vascular Aβ-deposition was observed. For subclassification of CAA types, we noted whether capillary Aβ-deposits were present. Because the subiculum-CA1 region, the entorhinal cortex, and the occipital cortex (Brodmann areas 17, 18, and 19) are predilection sites for capillary CAA [69], these regions were screened for capillary CAA. In the event that capillary Aβ-deposition was observed even in a single capillary in one of the above-mentioned regions, cases were classified as CAA-type 1 (capillary CAA), whereas those lacking capillary Aβ but having Aβ-deposits in arteries or veins were referred to as CAA-type 2 [68].

Reference pathology for all cases was performed by one and the same neuropathologist (DRT).

Table 1 AD and non-AD cases: distribution of age, gender, Braak NFT-stage for AD-related neurofibrillary pathology [12], Aβ-phase [72], CERAD-score for neuritic plaques [49], and CAA-type [68]

Age	50–60	61–70	71–80	81–90	91–100	101–110	
Non-AD controls	4.85 (15)	30.74 (95)	38.83 (120)	21.36 (66)	3.88 (12)	0.32 (1)	
AD	1.41 (1)	11.27 (8)	30.99 (22)	39.44 (28)	15.49 (11)	1.41 (1)	
Gender	Male	Female					
Non-AD controls	59.22 (183)	40.78 (126)					
AD	32.39 (23)	67.61 (48)					
Braak-stage	0	I	II	III	IV	V	VI
Non-AD controls	7.44 (23)	33.33 (103)	36.25 (112)	19.74 (61)	2.59 (8)	0.32 (1)	0.32 (1)
AD	0 (0)	0 (0)	0 (0)	5.63 (4)	30.99 (22)	36.62 (26)	26.76 (19)
Aβ-phase	0	1	2	3	4		
Non-AD controls	30.10 (93)	17.80 (55)	22.98 (71)	20.06 (62)	9.06 (28)		
AD	0 (0)	1.41 (1)	2.82 (2)	22.54 (16)	73.24 (52)		
CERAD-score	0	1	2	3			
Non-AD controls	80.82 (236)	9.93 (29)	5.48 (16)	3.77 (11)			
AD	1.41 (1)	12.68 (9)	29.58 (21)	56.34 (14)			
CAA-type	0	1	2				
Non-AD controls	57.28 (177)	15.53 (48)	27.18 (84)				
AD	7.04 (5)	56.34 (40)	36.62 (26)				

The numbers provide the percentage of non-demented elderly as well as AD cases. The total numbers are presented in parenthesis. The gender distribution confirms the previously reported higher risk for women to develop AD (Fisher's exact test: $p < 0.001$) [18, 23]

Table 2 Concurrent neurological disorders of non-AD cases

None	259
Mixed dementia	16
Vascular dementia	12
Dementia with Argyrophilic grain disease	12
Parkinson/dementia with Lewy bodies	1
Schizophrenia	1
Progressive multifocal leukencephalopathia	1
Non-AD dementia NOS	7

Non-AD controls included cognitively normal individuals as well as the 50 cases with non-AD dementia listed here. They include one Braak NFT-stage V and one Braak NFT-stage VI cases exhibiting severe Parkinson's disease pathology corresponding to neuropathological PD stages 5 and 6 according to Braak et al. [14] categorized as mixed dementia cases. Statistical comparisons are depicted in Table 5

NOS not otherwise specified

In 18 AD and 70 control cases, we rated the overall CAA distribution according to CAA-stages [67]. For this purpose, CAA was also studied in anti-$A\beta_{17-24}$- or Campbell–Switzer-stained sections of the basal ganglia, thalamus, and cerebellum.

Genotyping

Genomic DNA was extracted either from unfixed frozen brain tissue or from the paraffin-embedded brain tissue [24]. For high-quality genomic DNA templates, *APOE* genotyping was performed by PCR followed by digestion with the restriction enzyme *HhaI* [32]. For DNA templates from formaldehyde-fixed specimens, a reliable semi-nested PCR protocol was employed to achieve high yields of PCR-product [24].

The polymorphisms in apoE- and cholesterol metabolism-related genes listed in Table 3 and discussed as AD risk factors [40, 44, 45, 54, 55] were analyzed for potential association with the CAA types in relation to AD. Owing to the common low-quality retrieval of genomic from formaldehyde-fixed archival tissue samples, PCR protocols that yield shorter products were adapted to genotype both cholesterol 25-hydroxylase gene (*CH25H*) polymorphisms: A list of these primers and restriction enzymes is supplied in Table 4.

Immunohistochemistry

Abnormal phosphorylated τ-protein was visualized with a monoclonal antibody (AT-8, Innogenetics, Belgium, 1/1,000) and Aβ-plaques using an antibody raised against $A\beta_{17-24}$ (Covance, Emeryville, CA, USA: 4G8 [42], 1/5,000, formic acid pretreatment). EAAT-1 (Novocastra, Newcastle, UK: 10D4 [60], 1/100, 24 h at 22°C, microwave pretreatment), EAAT-2 (Novocastra, Newcastle, UK: 1H8 [48], 1/40, 24 h at 22°C, microwave pretreatment), and apoE (Covance: D6E10 [70], 1/500, 24 h at 22°C, microwave and formic acid pretreatment) were detected using monoclonal

Table 3 List of genetic polymorphisms studied

Gene	Polymorphism	Description in AD	Genotyping protocol	CAA-1	CAA-2	AD-capCAA	AD-other
APOE (apolipoprotein E)	ε4 allele	[19]	[24]	$p < 0.001$ OR: 3.801 CI: 2.362–6.117 $n = 302$ (58 CAA-1 + 244 non-CAA-1)	$p = 0.832$ $n = 302$ (92 CAA-2 + 210 non-CAA-2)	$p < 0.001$ OR: 3.77 CI: 1.973–7.204 $n = 311$ (24 AD + 287 controls)	$p = 0.708$ $n = 310$ (23AD + 287 controls)
CYP46A1 (cholesterol 24-hydroxylase)	rs754203	[55]	[55]	$p = 0.253$ $n = 205$ (42 CAA-1 + 163 non-CAA-1)	$p = 0.617$ $n = 205$ (58 CAA-2 + 147 non-CAA-2)	$p = 0.349$ $n = 197$ (21 AD + 176 controls)	$p = 0.887$ $n = 196$ (20 AD + 176 controls)
CYP46A1 (cholesterol 24-hydroxylase)	rs4900442	[45]	[45]	$p = 0.999$ $n = 62$ (15 CAA-1 + 47 non-CAA-1)	$p = 0.395$ $n = 62$ (20 CAA-2 + 42 non-CAA-2)	$p = 0.604$ $n = 58$ (8 AD + 50 controls)	$p = 0.509$ $n = 54$ (4 AD + 50 controls)
CYP46A1 (cholesterol 24-hydroxylase)	rs7157609	[44]	[44]	$p = 0.502$ $n = 176$ (34 CAA-1 + 142 non-CAA-1)	$p = 0.050$ $n = 176$ (53 CAA-2 + 123 non-CAA-2)	$p = 0.247$ $n = 163$ (19 AD + 144 controls)	$p = 0.237$ $n = 163$ (19 AD + 144 controls)
*CH25H**1 (cholesterol 25-hydroxylase)[a]	6,443 bp (rs13500)	[54]	[54]	$p = 0.533$ $n = 201$ (42 CAA-1 + 159 non-CAA-1)	$p = 0.443$ $n = 201$ (55 CAA-2 + 146 non-CAA-2)	$p = 0.205$ $n = 192$ (20 AD + 172 controls)	$p = 0.564$ $n = 192$ (20 AD + 172 controls)
*CH25H**2 (cholesterol 25-hydroxylase)[a]	6,627 bp (rs1131706) T-allele	[54]	[54]	$p = 0.013$ OR: 3.957 CI: 1.334–11.743 $n =$ (42 CAA-1 + 159 non-CAA-1)	$p = 0.876$ $n = 201$ (55 CAA-2 + 146 non-CAA-2)	$p = 0.186$ $n = 192$ (20 AD + 172 controls)	$p = 0.485$ $n = 192$ (20 AD + 172 controls)
LRP-1 ($\alpha_2$macroglobulin receptor/low-density lipoprotein receptor-related protein-1)	C766T T-allele	[17, 40]	[25]	$p = 0.493$ $n = 166$ (35 CAA-1 + 131 non-CAA-1)	$p = 0.236$ $n = 166$ (50 CAA-2 + 116 non-CAA-2)	$p = 0.289$ $n = 148$ (20 AD + 128 controls)	$p = 0.037$ OR: 2.851 CI: 1.063–7.649 $n = 146$ (18 AD + 128 controls)

CAA-1, shows the associations between CAA-type 1 cases with the genetic polymorphisms in contrast to non-CAA-type 1 cases regardless of the clinical diagnosis of AD; CAA-2 shows the associations between CAA-type 2 cases with the genetic polymorphisms in contrast to non-CAA-type 2 cases regardless of the clinical diagnosis of AD; AD with capCAA displays the association of genetic polymorphisms with AD cases with CAA-type 1 (capillary CAA) in contrast to all other cases. AD without capCAA displays the association of genetic polymorphisms with all AD cases without CAA-type 1 (capillary CAA) in contrast to all other cases

Due to the use of paraffin-embedded archival material not all polymorphisms could be determined in all cases. This explains the variations in the number of cases

[a] *CH25H**1 and *CH25H**2 were studied in one model term controlled for age and gender

antibodies. The primary antibodies were detected with a biotinylated secondary antibody and the ABC complex (Vectastain: Vector Laboratories, Burlingame, CA, USA), and this reaction was subsequently visualized with 3,3-diaminobenzidine (DAB). Immunolabeled paraffin sections were counterstained with hematoxylin. PEG-sections were not counterstained. Positive and negative controls were included.

In selected cases, double immunolabeling was performed to demonstrate the spatial relationship between EAAT-2, glial fibrillary acidic protein (GFAP) expression, and vascular Aβ-deposition. EAAT-2 was visualized with

Table 4 List of primer pair sequences employed to genotype formaldehyde-fixed archival tissue samples

Gene	SNP	Primer pairs (Sequence: 5′ to 3′)	PCR-product	Restriction enzyme	DNA fragments
CYP46A1	rs7157609	F: CGGACCTGAGTCTGAAGAGG	319 bp	*Mbo*II	A: 293 + 26
	[A/G]	R: CGAGCCGACTCAGCTGTC			G: 238 + 55 + 26
	rs754203	F: CAACCCTATTCCATGGCTGT	220 bp	*Msp*I or	C: 121 + 99
	[C/T]	R: GACCCGAAGGAAACTGTCAA		*Hpa*II	T: 220
CH25H	CH25H*1 [C/T]	F: CCTGCTTCACGTCCCTATGT	172 bp	*Msp*I or	C: 96 + 76
	(rs13500)	R: CGCCCTGCCTATATTAACCA		*Hpa*II	T: 172
	CH25H*2 [A/T]	F: CATCTGTGTGAAGCCAAAGC	17 bp	*Bfa*I	A: 124 + 46
	(rs1131706)	R: GGGACGTGAAGCAGGTGTAT			T: 170

bp Base pairs, *F* forward, *R* reverse, *SNP* single nucleotide polymorphism

the monoclonal mouse IgG-antibody 1H8, GFAP with a polyclonal rabbit IgG-antibody (1/1,000, DAKO, 24 h at 22°C) or with a monoclonal antibody (1/20, G-A-5, Boehringer–Mannheim, 24 h at 22°C), and Aβ with a polyclonal antibody directed against $A\beta_{N1D}$ (1/100, [58], 24 h at 22°C, microwave and formic acid pretreatment). Antibodies directed against the N-terminus of Aβ have been shown to stain all vascular Aβ-deposits just as C-terminus-specific anti-Aβ-antibodies [64, 68]. One primary antibody was detected with a carbocyanine 2-labeled secondary antibody specifically directed against either mouse or rabbit IgG (Dianova, Hamburg, Germany), whereas the second primary antibody was detected using a carbocyanine 3-labeled secondary antibody specifically directed against either mouse or rabbit IgG (Dianova, Hamburg, Germany). All tissue sections were viewed with a Leica DMLB fluorescence microscope. Digital photographs were obtained with a Leica DC 500 camera and were edited for publication layout with the assistance of CorelPhotopaint® software, release 12.0.

Morphological analysis

11 AD and 14 control cases were studied for the astroglial expression of EAAT-2 in association with CAA-type 1 and 2. The selection of eight CAA-type 1 ($n = 2$ controls, 6 AD), nine CAA-type 2 cases ($n = 4$ controls, 5 AD), and eight cases without CAA was necessary as the EAAT-1 and EAAT-2 antibodies are ineffective on long-time formalin-fixed material (data not shown) but do function on short-time fixed material [48, 65]. Therefore, we used cases that were fixed no longer than 3 weeks prior to paraffin embedding. To ensure a similar staining quality, only cases from a single center (Offenbach am Main, Germany) were used to study EAAT-1 and EAAT-2 expression. For semiquantitative estimation of the relationship between EAAT-2-expressing astrocytes and blood vessels in the occipital cortex, 20 cortical vessels in layers II and III were studied for association with EAAT-2 immunopositive astrocytic processes. For vessel selection, a region of interest (ROI) in Brodmann area 17 was chosen randomly. All blood vessels found there in layers II–III were consecutively examined until a total of 20 vessels were evaluated. Association of EAAT-2-positive astrocytes with a blood vessel was recorded when EAAT-2-positive processes had contact with the perivascular glia limitans of a given vessel. Four degrees were differentiated: 0 = no visible association; 1 = association with less than 33.3% of the vessel wall circumference showing EAAT-2-positive processes; 2 = association with 33.3–66.6% of the vessel wall circumference showing EAAT-2-positive processes; 3 = association with more than 66.6% of the vessel wall circumference showing EAAT-2-positive processes. The degree of association between EAAT-2-positive astrocytes and cortical blood vessels was obtained for each of the 20 vessels, and the scores were summed into one representative semiquantitative score.

Statistical analysis

Binary logistic regression analysis was used to estimate the association of genetic variables with CAA pathology and with distinct groups of cases controlling age and gender as covariates except otherwise mentioned [1]. Odds-ratios (OR) were obtained with 95% confidence intervals (CI). Computations were performed with the help of the SPSS® software, release 16.0.1.

Results

Our major finding was the distinction between two groups of AD cases based on morphological and genetic criteria. The morphological criteria were (1) presence or absence of capillary Aβ-deposition, (2) more versus less widespread senile plaque pathology, and (3) reduction or no reduction of perivascular EAAT-2 expression in the Brodmann area 17 (occipital cortex). Genetically, capillary CAA-related AD was associated with the *APOE* ε4 allele, whereas other AD cases exhibited an increased frequency of the *LRP-1*

C766T T-allele. Detailed statistical analyses are shown in Tables 3 and 5.

Morphological distinction between AD cases with and without capillary Aβ-deposition

In addition to capillary Aβ-deposition, AD cases with capillary CAA displayed more widely distributed Aβ-plaque deposits, as represented by the phase of Aβ-deposition in the medial temporal lobe (MTL), than other AD cases ($p = 0.006$; further information on the statistical analysis is provided in Table 5), whereas the Braak NFT-stage ($p = 0.886$) and the CAA-stage did not differ ($p = 0.08$).

Compared to controls, AD cases with capillary CAA displayed more widespread neurofibrillary tangle pathology (Braak NFT-stage: $p < 0.001$), neuritic plaques, as represented by the CERAD-score ($p < 0.001$), Aβ-plaques ($p < 0.001$), and CAA ($p = 0.002$) (Tables 5, 6). Immunohistochemistry using an antibody directed against apoE demonstrated that capillary Aβ-deposits in CAA-type 1 cases exhibited strong immunolabeling (Fig. 1a, b). Such an accumulation of apoE along capillaries was not seen in AD cases lacking capillary CAA. CAA-involved arteries and veins, however, were immunostained with anti-apoE in both CAA-type 1 as well as CAA-type 2 cases regardless of the apoE genotype.

Our analysis of astroglial EAAT-1 and EAAT-2 expression in the occipital cortex revealed no changes in EAAT-1 expression either in AD cases with and without CAA-type 1 or controls (Fig. 1d). In control individuals, cortical blood vessels were frequently rimmed by EAAT-2-positive processes of perivascular astrocytes (Fig. 1c). AD cases with capillary CAA, i.e., CAA-type 1, as well as controls with CAA-type 1 exhibited reduced numbers of EAAT-2-positive cell processes around blood vessels in the occipital cortex compared to controls and CAA-type 2 AD cases (Mann–Whitney U test: $p = 0.047$) (Fig. 2). Double label immunohistochemistry showed the colocalization of EAAT-2 and GFAP in perivascular astrocytes of controls and in AD cases with CAA-type 2 (Fig. 1e–g), whereas astrocytes surrounding CAA-affected capillaries in AD cases with CAA-type 1 expressed GFAP but often failed to express EAAT-2 (Fig. 1h–m).

Compared to controls, AD cases that lacked capillary CAA showed more widespread neurofibrillary tangle pathology, neuritic plaques, as represented by the CERAD-score, and Aβ-plaques (all: $p < 0.001$) but did not differ significantly from controls with regard to the distribution of CAA-affected vessels (CAA-stage; $p = 0.116$) (Table 5). This group of AD cases contained cases with CAA-type 2 and cases without CAA.

Genetic distinction between AD cases with and without capillary Aβ-deposition

AD cases with capillary CAA were strongly associated with the *APOE* ε4-allele in comparison with other AD cases (Logistic regression analysis: OR = 4.751, CI = 1.551–

Table 5 Odds-ratio (OR), confidence intervals (CI), p values of binary logistic regression analysis, and the number of observed cases in a given analysis (n)

Parameter	p	OR	CI	n
Comparison between AD cases with and without capillary CAA				
Braak-stage	0.886			71
Aβ phase	0.006	4.454	1.536–12.918	71
CERAD-score	0.652			71
CAA-stage	0.08			18
CDR-score	0.843			47
Comparison between AD cases with capillary CAA (CAA-type 1) and non-AD controls				
Braak-stage	<0.001	29.259	9.098–94.092	349
Aβ phase	<0.001	14.555	5.934–35.704	349
CERAD-score	<0.001	6.73	3.873–11.693	332
CAA-stage	0.002	6.220	1.987–19.469	81
Comparison between AD cases lacking capillary CAA and non-AD controls				
Braak-stage	<0.001	16.008	5.815–44.069	340
Aβ phase	<0.001	4.513	2.565–7.939	340
CERAD-score	<0.001	6.003	3.77–9.557	323
CAA-stage	0.116			77

All terms were controlled for age and gender. OR and CI were provided only in the event that a given parameter shows significant differences in a comparison

Table 6 AD subtypes: distribution of age, gender, Braak-stage, Aβ-phase, and CDR-score

Age	50–60	61–70	71–80	81–90	91–100	101–110	
AD with capillary CAA	0 (0)	7.5 (3)	35 (14)	42.5 (17)	15 (6)	0 (0)	
AD without capillary CAA	3.23 (1)	16.13 (5)	25.81 (8)	35.48 (11)	16.13 (5)	3.23 (1)	
Gender	Male	Female					
AD with capillary CAA	35 (14)	65 (26)					
AD without capillary CAA	29,03 (9)	70,97 (22)					
Braak-stage	0	I	II	III	IV	V	VI
AD with capillary CAA	0 (0)	0 (0)	0 (0)	2.5 (1)	35 (14)	42.5 (17)	20 (8)
AD without capillary CAA	0 (0)	0 (0)	0 (0)	9.68 (3)	25.81 (8)	29.03 (9)	35.48 (11)
Aβ-phase	0	1	2	3	4		
AD with capillary CAA	0 (0)	0 (0)	0 (0)	15 (6)	85 (34)		
AD without capillary CAA	0 (0)	3.23 (1)	6.45 (2)	32.26 (10)	58.06 (18)		
CERAD-score	0	1	2	3			
AD with capillary CAA	2.5 (1)	12.5 (5)	25 (10)	60 (24)			
AD without capillary CAA	0 (0)	12.90 (4)	35.48 (11)	51.61 (16)			
CDR-score	0	0.5	1	2	3		
AD with capillary CAA	0 (0)	4 (1)	8 (2)	8 (2)	80 (20)		
AD without capillary CAA	0 (0)	0 (0)	13.64 (3)	0 (0)	86.36 (19)		

AD subtypes: Distribution of age, gender, Braak NFT-stage [12], Aβ-phase [72], CERAD-score for neuritic plaques [49], and CDR-score [51]. The classification of AD was based upon the presence or absence of capillary CAA. CDR-scores were available from 47 AD cases. All other parameters were obtained in all 71 AD cases. The numbers provide the percentage of AD cases with and without capillary CAA. The total numbers of cases are presented in parenthesis. Statistical comparisons are depicted in Table 5

14.555, $p = 0.006$, $n = 47$). In addition to *APOE*, the *LRP-1* C766T polymorphism made it possible to distinguish between AD cases with and without capillary CAA (Logistic regression analysis: OR = 0.101, CI = 0.018–0.613, $p = 0.012$, $n = 38$). The frequency of the T-allele was higher in AD cases lacking capillary CAA than in AD cases with capillary CAA ($p = 0.012$) and in control individuals ($p = 0.037$, Table 3). The distribution of the *APOE* ε4-allele was similar for AD cases lacking capillary CAA and for controls (Table 3). On the other hand, the *LRP-1* C766T polymorphism was not associated with AD in the presence of capillary CAA (Table 3).

Finally, the *CH25H**2 T-allele was associated with the occurrence of CAA-type 1 in general ($p = 0.013$, Table 3) but failed to reach significance when AD cases with and without capillary CAA were compared (Logistic regression analysis: $p = 0.064$, $n = 40$). A summary of further results is supplied in Table 3.

Distribution of CAA-type 1 and CAA-type 2 in AD and non-AD cases

As previously shown [69], 56.3% of the AD cases showed CAA-type 1, 36.6% CAA-type 2 and only 7.1% did not exhibit any CAA pathology (AD cases $n = 71$). In contrast, 57.3% of the age-related non-demented patients were free of CAA. Only 27.2% had CAA-type 2 and CAA-type 1 was seen even less frequently in only 15.5% (non-AD cases $n = 309$) (Tables 1, 2).

Demented non-AD cases with mixed dementia, vascular dementia, argyrophilic grain disease, Parkinson's disease, and other causes of dementia had distributions of CAA and its subtypes that were similar to controls with CAA-type 1 in 20%, CAA-type 2 in 30%, and no CAA in 50% of cases.

No association of a distinct CAA-type with brain infarction or hemorrhage

No association existed between a given CAA-type and the presence or absence of brain infarction or cerebral hemorrhage when comparing CAA-type 1 and 2 cases in the entire sample (hemorrhage: $p = 0.935$; infarction: $p = 0.483$) as well as restricted to the group of the AD cases (hemorrhage: $p = 0.999$; infarction: $p = 0.487$).

Discussion

Here, we demonstrate that the previously reported two types of CAA [68] differentiate not only between CAA cases but also between at least two types of "sporadic" AD (Fig. 3): AD cases with capillary CAA constitute a specific subtype that can be distinguished from other AD cases lacking capillary CAA. This subtype is further characterized by (1) a more widespread expansion of Aβ-plaque pathology as represented by the phase of Aβ-plaque distribution in the MTL, (2) a capillary

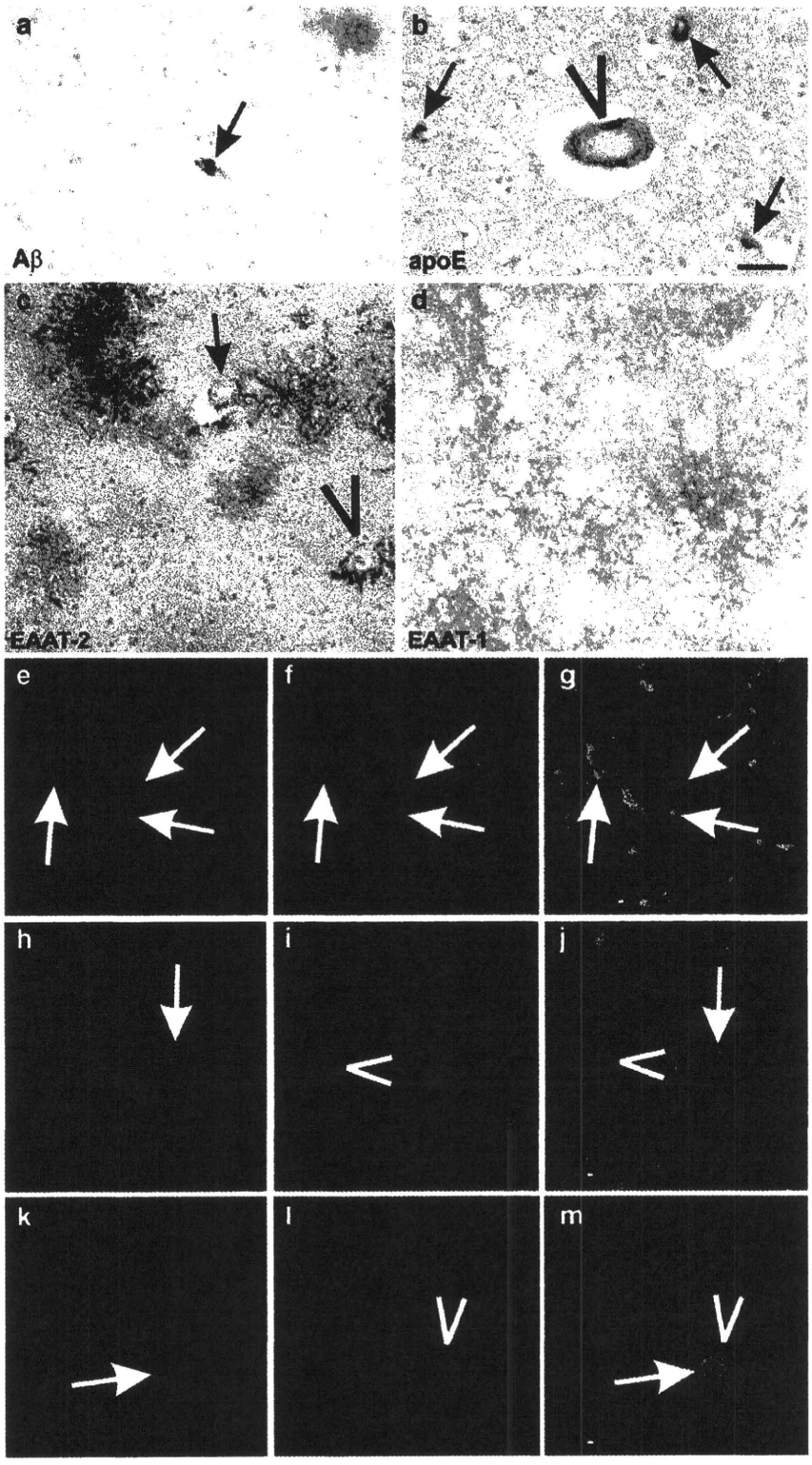

Fig. 1 **a** Capillary Aβ-deposition (*arrow*) in the temporal neocortex (Staining: anti-$A\beta_{17-24}$). **b** In a subsequent section of the temporal neocortex of the same case apoE was found in vascular Aβ-deposits: in cortical arteries (*arrowhead*) and cortical capillaries (*arrows*) (Staining: anti-human apoE). **c** Astrocytes expressing the glutamate transporter EAAT-2 in a control case free of any Aβ or τ-pathology show a circular (*arrows*) or mostly circular (*arrowhead*) perivascular expression in the cortex (Staining: anti-EAAT-2). **d** EAAT-1 is diffusely expressed in the cortex (Staining: anti-EAAT-1; control case free of Aβ and τ-pathology). **e–g** Perivascular EAAT-2 expression in astrocytes is seen in association with cortical blood vessels of an AD case without capillary CAA (**f**). The astroglial nature of the EAAT-2-positive cells is confirmed by the colocalization of GFAP (**e**, *arrows* in **e–g**) (Staining: anti-GFAP–anti-EAAT-2). **h–j** In an AD case with capillary CAA, capillary Aβ-deposits (*arrow* in **h**, **j**) were not associated with EAAT-2-positive astrocytes (**j**) whereas distant from the affected capillary EAAT-2-positive astrocytes were visible (arrowhead in **i**, **j**) (Staining: anti-$A\beta_{N1D}$–anti-EAAT-2). **k–m** Double label immunofluorescence demonstrates that perivascular astrocytes (*arrowhead*) near capillary Aβ-deposits (*arrow*) still express GFAP indicating the loss of EAAT-2 expression but not that of astrocytic processes (Staining: anti-$A\beta_{N1D}$–anti-GFAP). *Calibration bar* corresponds to 40 μm

CAA-related alteration of EAAT-2 expression in perivascular astrocytes of the occipital cortex, and (3) a robust association with the *APOE* ε4-allele as previously reported for capillary CAA regardless of AD [68]. Intracellular Aβ accumulation is also associated with the APOE ε4-allele [16].

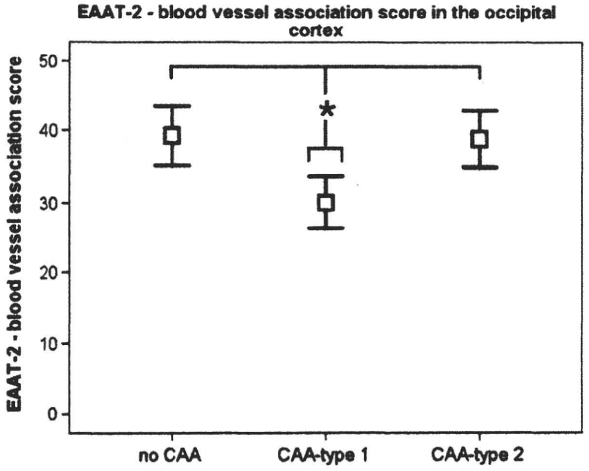

Fig. 2 Perivascular processes of cortical astrocytes less frequently exhibit the glutamate transporter EAAT-2 in cases with capillary CAA (CAA-type 1) than in those without as indicated by a lower semiquantitative perivascular EAAT-2 expression score in cases with capillary CAA than in those without CAA or with CAA-type 2 (*Asterisk* represent Mann–Whitney *U* test: $p < 0.05$)

Our second group of AD cases that lacked capillary Aβ-deposition and alterations of perivascular astrocytes, as indicated by EAAT-2 expression failure, included AD cases with CAA-type 2 and AD cases without CAA, and was associated with the presence of the T-allele of the *LRP-1* C766T polymorphism instead of with that of the *APOE* ε4-allele. Moreover, a distinction between *APOE* ε4-AD cases and non-ε4 AD cases appears to be important for treatment efficacy as well [59]. Aβ-targeting treatment outcomes (Phase II bapineuzumab study) differ based on *APOE* ε4 status, whereby *APOE* ε4-negative AD patients responded more favorably to treatment than *APOE* ε4 carriers [59]. These findings are consistent with the idea that *APOE* ε4 carriers frequently develop a distinct subtype of AD that is substantially different from other AD cases.

Alternatively, one could assume that capillary CAA and widespread Aβ-plaque-deposition in *APOE* ε4-carriers may represent the most severe degree of AD-pathology rather than a distinct type of AD. Were this assumption to be true, *APOE* ε4-carriers should not differ from other cases in earlier AD stages morphologically and genetically apart from their *APOE* ε4 status. Nonetheless, three counterarguments strongly contradict this viewpoint: (1) In our sample, capillary CAA in *APOE* ε4-carriers was already evident in early disease stages (Aβ-phase 1) [68, 69], thereby indicating that capillary CAA represents a distinct type of pathology rather than simply the end stage of Aβ-deposition. (2) One explanation for the development

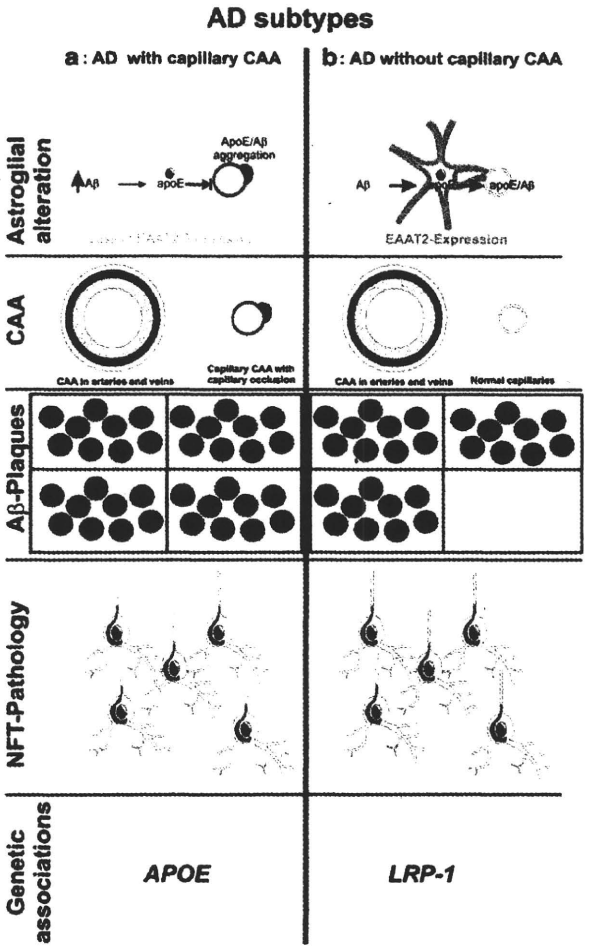

Fig. 3 Morphological and genetic characteristics of the two subtypes of sporadic AD. **a** AD with capillary CAA: Perivascular astrocytes lack EAAT-2 expression. CAA is not only seen in arteries and veins but also occurs in cortical capillaries and leads to capillary occlusion. Aβ-plaques are more widely distributed throughout the entire brain than in other AD cases (indicated by the number of fields showing Aβ-plaques and representing different brain regions). NFT-pathology is widely distributed and constitutes the diagnosis of AD. There is a strong association with the *APOE* ε4-allele. **b** AD without capillary CAA: This second group of AD cases does not show astroglial alterations or capillary CAA. Aβ-plaques are less widely distributed than in *APOE* ε4-related AD cases (indicated by a single field displaying no plaques representing a brain region that contains Aβ-plaques in AD cases with capillary CAA but not in those without capillary CAA). The neurofibrillary pathology as indicated by the Braak NFT-stage constitutes the diagnosis of AD and does not differ significantly from that of AD cases with capillary CAA. Genetically, AD cases lacking capillary CAA did not show an association with the *APOE* ε4 allele but with the T-allele of the *LRP-1* C766T polymorphism. It is not clear whether this group of AD cases that includes cases with and without CAA can be assigned as one group of cases or whether further subtypes can be identified in the future covering cases of this group

of capillary CAA is provided by different properties of apoE2, apoE3, and apoE4 in the transendothelial clearance of apoE-Aβ-complexes at the capillary segment of the BBB [20]. Here, endothelial transcytosis of apoE4-Aβ complexes was reduced when compared to that of apoE2-Aβ and apoE3-Aβ complexes [20]. Thus, it is reasonable to hypothesize that apoE4-Aβ complexes are less sufficiently cleared into the blood and, therefore, accumulate in the capillary vessel wall resulting in capillary CAA. (3) AD cases that lack capillary CAA differ genetically from AD cases having capillary CAA and from controls with respect to the frequency of the *LRP-1* C766T T-allele. This, too, points to the greater likelihood that AD cases without capillary CAA, and frequently not carrying an *APOE* ε4-allele but the T-allele of the *LRP-1* C766T polymorphism, constitute a genetically distinct class of individuals. Thus, our data strongly point to the existence of at least two specific AD subtypes related to CAA.

In cases with capillary CAA, cortical perivascular astrocytes often lack EAAT-2 expression, especially around capillaries with Aβ-deposits. Whether hypoperfusion traceable to CAA-related capillary occlusion impairs these astrocytes [66] or whether apoE-Aβ complexes directly impact on these cells during clearance is not yet known. However, Arg-61 apoE mice, a transgenic mouse model mimicking human apoE4 function by exhibiting similar interactions between apoE protein domains as apoE4, showed synaptic pathology as well as reduced levels of EAAT-2 (GLT-1) expression in astrocytes [87]. This apoE4-like domain interaction was also associated with a higher vulnerability to endoplasmatic reticulum (ER)-stress [86]. Thus, an apoE4-related effect may provide an alternative explanation for the alteration of perivascular astrocytes in AD cases with capillary CAA. This hypothesis is supported by the finding that astrocytes with a reduced EAAT-2 expression exhibit increased levels of cholesterol 24S-hydroxylase indicating alterations in the cerebral cholesterol metabolism [77] possibly influenced by apoE4-related alterations in cholesterol transport. Whether the reduction of EAAT-2 expression in perivascular astrocytes results in a general dysfunction of these cells or in excitotoxicity is currently unclear. One could speculate that dysfunction of perivascular astrocytes that constitute the glia limitans, which is the border between the perivascular space and the brain parenchyma, may contribute to BBB alterations. An argument favoring astroglia-related BBB alterations in capillary CAA cases is the finding that CAA reduces the expression of water (aquaporin 4) and potassium channels (Kir. 4) in astrocytic glia limitans end feet [84], presumably resulting in imbalances of the water and potassium distribution. Since the glutamate concentration in the AD brain declines [34], it seems less likely that reduced glutamate transporter expression has a major impact on excitotoxicity. An additional aspect in support of this hypothesis is that no differences exist in the NFT-distribution in the brain between AD cases with and without capillary CAA, although excitotoxicity is capable of influencing τ-pathology [34, 61] that represents the molecular basis of NFTs.

AD cases without capillary CAA were not associated with the *APOE* ε4-allele but with the T-allele of the *LRP-1* C766T polymorphism. This polymorphism has been discussed previously as being associated with AD [40], but other authors could not replicate that group's findings [7, 8]. Inasmuch as the polymorphism was only associated with a distinct AD subtype, heterogeneity of study samples in previous analyses may explain the conflicting results. The T-allele of the *LRP-1* C766T polymorphism was related to the severity of CAA in arteries and veins but not to that in capillaries [17], thereby providing a possible explanation for its association with AD cases lacking capillary CAA. The possession of an *LRP-1* C766T T-allele leads to an increased expression of LRP-1 [39] and to a reduction of Aβ-plaques in the brain. A possible explanation for this scenario is increased clearance of Aβ through this receptor either in its apoE-bound form [20, 43] or in its $\alpha_2$macroglobulin-bound form [52]. Because LRP-1 is expressed in perivascular astrocytes of the glia limitans [28, 78], a presumably increased LRP-1-related clearance of Aβ into the perivascular space [6, 81, 82] by T-allele carriers may lead to CAA formation. As such, non-capillary vessels would be the major target because capillary CAA formation can be prevented by transendothelial clearance of apoE2-Aβ and apoE3-Aβ complexes at the capillary segment [20]. Only APOE ε4-carriers with a less effective transendothelial clearance [20] are at high risk for capillary CAA. These results are also in line with our finding here of less advanced Aβ-plaque pathology in AD cases without capillary CAA and overrepresenting the *LRP-1* C766T T-allele than in those with capillary CAA. However, AD cases with and without capillary CAA displayed similar levels of NFT-pathology, thus indicating that possession of an *LRP-1* C766T T-allele may be beneficial for reducing amyloid deposition while, at the same time, permitting the development of neurofibrillary pathology. Since LRP-1 is a neuronal receptor [50] its increased expression in T-allele carriers [39] may support an influx of LRP-1 ligands, including Aβ, into neurons with hypothetic effects on τ-phosphorylation [53, 63]. Further detailed studies are required to clarify the pathogenesis of non-*APOE* ε4 AD without capillary CAA.

Other apoE and cholesterol-related genes studied here did not show a significant association with either type of AD. However, CAA-type 1 was associated with the T-allele of the *CH25H**2 polymorphism, thereby supporting the view that, for capillary CAA, alterations in cholesterol

transport (*APOE*) and metabolism (*CH25H*) are important contributors to the pathogenetic events. Notably, the pathogenesis of AD lacking capillary CAA can also be related to cholesterol transport and metabolism because the genetic association with the *LRP-1* C766T polymorphism points to the involvement of the apoE receptor LRP-1 in this subtype of AD.

Neither cerebral hemorrhage nor infarction was specifically associated with a distinct type of AD or even with CAA generally. These results confirmed those of a recently published autopsy study [4]. Thus, CAA-related hemorrhage and infarction cannot account for the differences between the two distinct AD subtypes, i.e., those with and without capillary CAA. Nor did the CDR-scores from both AD subtypes differ, thereby pointing to a very similar clinical phenotype.

Finally, we classified all demented cases with at least Braak NFT-stage III which exhibited significant levels of cerebrovascular or non-AD neurodegenerative pathology (e.g., argyrophilic grain disease, Parkinson's disease) as "mixed dementia" [37] or argyrophilic grain disease cases [13, 74]. We categorized these cases as well as cases with pure vascular dementia or dementia with Lewy bodies as demented non-AD cases. Because dementia results from overall brain damage, cerebrovascular and other neurodegenerative changes can lower the threshold for AD-related pathology so that these factors taken together result in dementia [22, 38, 74]. Such AD-related types of mixed dementia should be distinguished from "pure" AD cases with and without capillary CAA. In the event of multiple microinfarctions or argyrophilic grain disease, the clinical differentiation from pure AD cases may be very difficult, if not impossible. The inclusion of AD-related mixed dementia cases, especially in studies with large numbers of non-autopsy proven cases that fulfill clinical criteria for AD, may explain the existence of heterogeneous results from genetic association studies because the "demented non-AD" cases, clinically misdiagnosed as AD, exhibited CAA and its subtypes in a similar distribution pattern as control cases and were, therefore, indistinguishable from controls. In our sample of non-selected autopsy cases, there were 71 AD and 50 demented non-AD cases. This fact highlights the importance of the second group which although difficult to distinguish clinically from true AD cases, nevertheless represented 41% of our clinically demented cases.

In conclusion, a large cohort of autopsy cases provides a high phenotype resolution that permits identification of at least two specific types of AD which are not only morphologically distinct but also exhibit genetic and pharmacogenetic differences [59]. These differences include a distinction between *APOE* ε4 associated AD cases with capillary CAA versus *LRP-1* C766T T-allele-associated AD cases lacking capillary CAA. These results also imply that subtype specific treatment strategies must be developed, as suggested already by Salloway et al. [59].

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Brain Endothelial Cells Produce Amyloid β from Amyloid Precursor Protein 770 and Preferentially Secrete the *O*-Glycosylated Form*[S]

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Deposition of amyloid β (Aβ) in the brain is closely associated with Alzheimer disease (AD). Aβ is generated from amyloid precursor protein (APP) by the actions of β- and γ-secretases. In addition to Aβ deposition in the brain parenchyma, deposition of Aβ in cerebral vessel walls, termed cerebral amyloid angiopathy, is observed in more than 80% of AD individuals. The mechanism for how Aβ accumulates in blood vessels remains largely unknown. In the present study, we show that brain endothelial cells expressed APP770, a differently spliced APP mRNA isoform from neuronal APP695, and produced Aβ40 and Aβ42. Furthermore, we found that the endothelial APP770 had sialylated core 1 type *O*-glycans. Interestingly, O-glycosylated APP770 was preferentially processed by both α- and β-cleavage and secreted into the media, suggesting that *O*-glycosylation and APP processing involved related pathways. By immunostaining human brain sections with an anti-APP770 antibody, we found that APP770 was expressed in vascular endothelial cells. Because we were able to detect *O*-glycosylated sAPP770β in human cerebrospinal fluid, this unique soluble APP770β has the potential to serve as a marker for cortical dementias such as AD and vascular dementia.

Alzheimer disease (AD)[3] is characterized by intracellular accumulation of neurofibrillary tangles and extracellular deposits of amyloid β (Aβ) peptides in the brain (1, 2). Aβ is generated from amyloid precursor protein (APP) by the sequential actions of β-secretase, BACE1 (β-site APP-cleaving enzyme 1) (3–6), and γ-secretase (7). Because most early-onset familial AD patients have gene mutations that influence the processing or aggregation of Aβ, and the neurites associated with Aβ plaques are often damaged (2), the process of Aβ deposition in the brain seems to be strongly associated with AD pathogenesis. Even though APP has been proposed to have a receptor-like function and binds to multiple extracellular matrix proteins such as heparin and collagen (8, 9), understanding the biological functions of APP remains an important scientific and intellectual challenge (10). Two paralogs of APP are known in mammals and are designated APP-like proteins 1 and 2. Interestingly, triple knock-out mice lacking all three APP family members die shortly after birth (11), suggesting redundant functions of the APP family proteins.

APP has three alternatively spliced isoforms: APP695, APP751, and APP770 (12, 13). Compared with APP695, APP751 contains an additional Kunitz-type protease inhibitor (KPI) domain, whereas APP770 contains a KPI domain plus an OX2 domain. APP695 is most abundantly expressed in neurons, whereas APP751 and APP770 show more ubiquitous expression patterns (14). The secreted form of APP containing a KPI domain, also known as the protease nexin 2, potentially inhibits certain serine proteases, most notably several prothrombotic enzymes (15); very limited information is available concerning the functions of the OX2 domain. In the present study, we show that brain microvascular endothelial cells (BMECs) express significant levels of APP770, from which Aβ40 and Aβ42 are produced, and that the endothelial APP770 has multiple O-glycosylation chains, which potentially play important roles in APP processing.

EXPERIMENTAL PROCEDURES

Materials—The sources of the materials used in this study were as follows: tissue culture media and reagents including DMEM from Invitrogen; recombinant peptide *N*-glycosidase (PNGase) from New England BioLabs; *O*-glycosidase from Roche Applied Science; *Arthrobacter ureafaciens* sialidase from Nacalai Tesque; protein A-Sepharose Fast Flow from GE

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[3] The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid β-peptide; APP, amyloid precursor protein; BACE, β-site APP-cleaving enzyme; BMEC, brain microvascular endothelial cell; CSF, cerebrospinal fluid; KPI, Kunitz-type protease inhibitor; sAPP, soluble APP; PNA, peanut agglutinin (*Arachis hypogaea* lectin); PNGase, peptide-N glycosidase; APP-H, high molecular weight APP770; APP-L, low molecular weight APP770.