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N-truncated A β_{4-x} peptides in sporadic Alzheimer's disease cases and transgenic Alzheimer mouse models

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Abstract

Background: The deposition of neurotoxic amyloid- β (A β) peptides in plaques in the brain parenchyma and in cerebral blood vessels is considered to be a key event in Alzheimer's disease (AD) pathogenesis. Although the presence and impact of full-length A β peptides such as A β_{1-40} and A β_{1-42} have been analyzed extensively, the deposition of N-terminally truncated A β peptide species has received much less attention, largely because of the lack of specific antibodies.

Methods: This paper describes the generation and characterization of novel antibodies selective for A β_{4-x} peptides and provides immunohistochemical evidence of A β_{4-x} in the human brain and its distribution in the APP/PS1KI and 5XFAD transgenic mouse models.

Results: The A β_{4-x} staining pattern was restricted mainly to amyloid plaque cores and cerebral amyloid angiopathy in AD and Down syndrome cases and in both AD mouse models. In contrast, diffuse amyloid deposits were largely negative for A β_{4-x} immunoreactivity. No overt intraneuronal staining was observed.

Conclusions: The findings of this study are consistent with previous reports demonstrating a high aggregation propensity of A β_{4-x} peptides and suggest an important role of these N-truncated A β species in the process of amyloidogenesis and plaque core formation.

Keywords: Alzheimer's disease, N-truncated Amyloid- β , Immunohistochemistry, Mouse model, Postmortem, 5XFAD, APP/PS1KI

Background

Alzheimer's disease (AD) is a severe age-dependent neurodegenerative disorder accounting for the majority of dementia cases worldwide. It is characterized by two neuropathological hallmark lesions, namely the accumulation of amyloid- β (A β) peptides in the form of extracellular plaques and the formation of intracellular neurofibrillary tangles consisting of hyperphosphorylated tau protein [1]. The formation of extracellular plaques is mechanistically linked to the amyloid cascade hypothesis, positioning the

accumulation of A β peptides as a pivotal and triggering event in AD etiology [2].

The production of A β peptides depends on the sequential processing of the type I single transmembrane amyloid precursor protein (APP) by two proteases called β - and γ -secretase [3]. In addition to full-length A β_{1-40} and A β_{1-42} peptides, a variety of peptides with shorter C-termini, such as A β_{1-37} , A β_{1-38} , or A β_{1-39} , have been described [4, 5]. Furthermore, elongated amyloidogenic A β_{1-43} peptides have recently been reported [6]. Similar heterogeneity has been observed at the A β N-terminus, and several N-terminally truncated A β forms, including A β_{2-x} [7], A β_{pE3-x} [8, 9], A β_{5-x} [10, 11], and A β_{pE11-x} [9, 12, 13], have been demonstrated in parenchymal or vascular deposits of human AD brains by immunohistochemical methods. An

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even more complex pattern of A β peptides has been reported by the use of mass spectrometry [14–17], which detected further variants such as A β _{7–42}, A β _{8–42}, or A β _{9–42}. N-terminal truncations are believed to render A β peptides more neurotoxic and to promote enhanced peptide aggregation compared with the corresponding full-length peptides with an intact N-terminus [18].

A β _{4–x} peptides appear to be among the most abundant A β species, but not much is known about the spatial distribution of these N-terminally truncated peptides in AD brains. Already in 1985, Masters and colleagues [19] reported the presence of a major A β peptide species starting with the phenylalanine (Phe) at position 4 of the A β sequence in both AD and Down syndrome (DS) cases, an observation that was subsequently verified [20]. More recent mass spectrometric characterizations of A β isoform signatures in human brain samples confirmed that A β _{4–42} and A β _{1–42} represent highly abundant species in cortical samples of AD and control brains [14, 21]. Whether A β _{4–x} peptides also occur within intracellular compartments is currently unclear. Likewise, the origin of A β _{4–x} peptides remains unknown. In this respect, a recent *in vitro* study suggested that neurons predominantly secrete full-length A β peptides starting with the aspartate in position 1, whereas astrocytes and microglia in addition secrete measurable amounts of N-terminally truncated species, including A β _{4/5–x} [22]. The aim of the present work was to characterize newly generated A β _{4–x}-specific antibodies as a tool to study A β _{4–x} peptides in different AD transgenic mouse models and human patients with sporadic AD.

Methods

Development of A β _{4–x}-specific polyclonal antibodies in guinea pigs

Antibodies were generated by the Peptide Specialty Laboratory (PSL; Heidelberg, Germany). In brief, immunization was carried out in two guinea pigs using the six-amino acid peptide (FRHDSG) corresponding to residues (4–9) of the A β peptide and coupled at its C-terminus via an additional cysteine residue to maleimide-activated keyhole limpet hemocyanin (KLH) (Thermo Fisher Scientific, Waltham, MA, USA). Antisera were generated by injection of peptides conjugated to KLH and emulsified with complete Freund's adjuvant for a first injection, followed by injections 2, 3, and 4 at 2-week intervals with incomplete Freund's adjuvant. Serum was collected prior to the first immunization (preimmune serum) and after the fourth immunization. The immune sera obtained after the final bleed were affinity-purified and named 029-1 and 029-2.

Antibody characterization by capillary isoelectric focusing immunoassay and Western immunoblot analysis

To assess the selectivity of purified polyclonal antibodies 029-1 and 029-2 for different N-terminal variants of A β , we employed a capillary isoelectric focusing (CIEF) immunoassay [23] and urea-bicine/bis-tris/Tris/sulfate sodium dodecyl sulfate (SDS)-PAGE followed by Western immunoblotting [22, 24]. For the CIEF immunoassay, synthetic A β peptides with different N-termini were separated on a Peggy Sue device (ProteinSimple, San Jose, CA, USA) by isoelectric focusing in microcapillaries and subsequently were probed with purified 029-1 and 029-2 antibodies. For comparison, the CIEF immunoassay was also performed with the mouse monoclonal antibodies IC16 [25] and 6E10 (BioLegend, San Diego, CA, USA), which recognize several N-terminal variants of A β . The synthetic A β peptides A β _{1–40}, A β _{2–40}, A β _{3–40}, pyroglutamate A β _{pE3–40}, A β _{4–40}, and A β _{5–40} were purchased from AnaSpec (Fremont, CA, USA). Peptide stock solutions (1 mg/ml) were prepared in dimethyl sulfoxide (DMSO) or, in the case of A β _{pE3–40}, in 0.1% NH₄OH and stored at –80 °C. Aliquots were thawed only once. The peptides were further diluted in 20 mM bicine, pH 7.6, with 0.6% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate and then mixed 1:4 with Premix G2 containing a pH 5–8 nested gradient, fluorescent pH standards, and a DMSO inhibitor mix (all reagents were obtained from ProteinSimple). The automated CIEF immunoassay on the Peggy Sue platform (ProteinSimple) was carried out as described previously for the NanoPro platform (ProteinSimple) [23]. The final peptide concentration in the microcapillaries was 100 ng/ml per peptide. Assuming an internal capillary volume of roughly 0.5 μ l, this corresponded to approximately 50 pg of peptide per capillary. Primary antibodies 029-1, 029-2, 6E10, and IC16 were diluted in antibody diluent (ProteinSimple). Detection was achieved with biotinylated anti-guinea pig immunoglobulin G (IgG; Dianova, Barcelona, Spain) in combination with streptavidin-horseradish peroxidase (HRP) (ProteinSimple) for antibodies 029-1 and 029-2 or with goat-anti-mouse-HRP secondary antibody (ProteinSimple) for 6E10 and IC16.

For SDS-PAGE analysis, stock solutions of synthetic A β _{1–40}, A β _{2–40}, A β _{3–40}, pyroglutamate A β _{pE3–40}, and A β _{4–40} were prepared in sample buffer (0.36 M bis-tris, 0.16 M bicine, 15% wt/vol sucrose, 1% wt/vol SDS, and 0.0075% wt/vol bromophenol blue) and stored at –80 °C. The A β peptides were further diluted in sample buffer, and aliquots containing 0.1, 1, and 10 ng of peptide per lane were separated by urea-bicine/bis-tris/Tris/sulfate SDS-PAGE and analyzed by immunoblotting as previously described [7]. The blotting membranes were blocked in 2% electrochemiluminescence (ECL) advance blocking agent (GE Healthcare Life Sciences, Little

Chalfont, UK) in PBS with 0.1% Tween 20 (PBS-T) for at least 4 h at room temperature (RT) and probed with the primary antibody 029-2 (1:300 in PBS-T) at 4 °C overnight. After three washing steps with PBS-T, biotinylated goat anti-guinea pig IgG (1:3000 in PBS-T; Life Technologies, Carlsbad, CA, USA) and streptavidin-HRP were employed as secondary reagents. Chemiluminescent signals obtained with ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) were recorded. After three additional washing steps in PBS-T, the membrane was reprobed with 6E10 (1:1000 in PBS-T) and developed with biotinylated goat anti-mouse IgG (333 ng/ml in PBS-T; Vector Laboratories Ltd., Peterborough, UK) in combination with streptavidin-HRP complex (1:3000 in PBS-T).

Peptide competition assay

To further assess the selectivity of the A β_{4-x} -specific polyclonal antibodies, a peptide competition experiment was performed. Antibody solution 029-2 was incubated with an excess of synthetic A β_{1-40} or A β_{4-40} for 5 h at RT with constant agitation, followed by centrifugation at 14,000 rpm for 5 minutes. The supernatant was used for immunohistochemical analysis of transgenic 5XFAD mouse brain tissues.

Human brain samples

Human brain samples from sporadic AD ($n = 16$, mean age 84.18 ± 8.04 years), nondemented control subjects ($n = 6$, mean age 84.17 ± 5.19 years), and DS ($n = 2$, mean age 61.0 ± 4.24 years) were obtained from the Netherlands Brain Bank (Table 1). The present study was approved by the ethics committee of the University Medical Center Göttingen.

Alzheimer mouse models

The generation of APP/PS1KI mice has been described previously [26]. In this AD mouse model, human mutant APP751 containing the Swedish and London mutations is overexpressed under the control of the murine Thy-1 promoter, whereas murine presenilin-1 (PSEN1) with the M233T and L235P familial Alzheimer's disease (FAD)-linked mutations is expressed under the control of the endogenous mouse PSEN1 promoter. APP/PS1KI mice were a generous gift of Dr. Laurent Pradier, Sanofi-Aventis, Paris, France. The analysis of 5XFAD mice (Tg6799) has also been reported previously [27, 28]. 5XFAD mice overexpress APP695 carrying the Swedish, Florida, and London mutations under the control of the murine Thy-1 promoter. In addition, human PS1 carrying the M146L and L286V mutations is also expressed under the control of the murine Thy-1 promoter. Male mice on a C57BL/6 \times SJL genetic background were obtained from The Jackson Laboratory [strain B6SJL-

Table 1 Clinical and pathological data of sporadic Alzheimer's disease cases, Down syndrome cases, and nondemented control subjects

| Case | Age (years) | Braak stage | ApoE | A β_{4-x} (029-2) | | A β (IC16) | |
|---------------------------|-------------|-------------|------|-------------------------|-----|------------------|-----|
| | | | | SP | CAA | SP | CAA |
| Sporadic AD cases | | | | | | | |
| AD-1 | 87 | V | 3/3 | ++ | ++ | +++ | ++ |
| AD-2 | 83 | VI | 4/3 | + | + | +++ | + |
| AD-3 | 78 | V | 4/3 | ++ | + | +++ | ++ |
| AD-4 | 79 | IV | 4/3 | ++ | +++ | ++ | +++ |
| AD-5 | 82 | V | 4/4 | + | + | +++ | ++ |
| AD-6 | 84 | IV | 4/3 | ++ | ++ | +++ | +++ |
| AD-7 | 85 | VI | 3/3 | (+) | - | +++ | ++ |
| AD-8 | 92 | IV | 3/3 | + | + | ++ | + |
| AD-9 | 92 | IV | 4/2 | + | ++ | +++ | ++ |
| AD-10 | 73 | IV | 4/4 | + | ++ | +++ | +++ |
| AD-11 | 92 | IV | 3/3 | ++ | + | +++ | ++ |
| AD-12 | 93 | IV | 3/3 | +++ | (+) | +++ | (+) |
| AD-13 | 81 | IV | n.d. | ++ | ++ | +++ | +++ |
| AD-14 | 95 | IV | 3/3 | + | - | ++ | - |
| AD-15 | 91 | IV | 4/3 | + | + | +++ | + |
| AD-16 | 65 | VI | 3/3 | (+) | - | ++ | - |
| AD-17 | 79 | V | 3/3 | + | + | ++ | + |
| Nondemented control cases | | | | | | | |
| NDC-1 | 87 | II | n.d. | - | - | - | - |
| NDC-2 | 90 | I | 2/2 | + | + | ++ | ++ |
| NDC-3 | 79 | II | 4/3 | + | - | ++ | - |
| NDC-4 | 89 | III | n.d. | - | + | - | + |
| NDC-5 | 78 | I | 3/3 | - | + | (-) | - |
| NDC-6 | 82 | I | 3/3 | - | - | - | - |
| Down syndrome | | | | | | | |
| DS-1 | 64 | V | 3/3 | ++ | ++ | +++ | +++ |
| DS-2 | 58 | VI | 4/3 | ++ | +++ | +++ | +++ |

Abbreviations: A β Amyloid- β , AD Alzheimer's disease, ApoE Apolipoprotein E, CAA Cerebral amyloid angiopathy, DS Down syndrome, NDC Nondemented control subject, SP Senile plaques

The following semiquantitative scoring criteria were used: - no staining, (+) barely detectable staining, + weak staining, ++ moderate staining, +++ extensive staining

Tg(A β PPSwFLon,PSEN1*M146L*L286V)6799Vas/J; The Jackson Laboratory, Bar Harbor, ME, USA] and backcrossed for ten generations to C57BL/6 J wild-type mice to obtain an incipient congenic line on a C57BL/6 J genetic background. All animals were handled according to German guidelines for animal care.

Immunohistochemical and immunofluorescence analyses

Mice were killed by CO₂ anesthetization followed by cervical dislocation. Brain samples were dissected and postfixed in 4% phosphate-buffered formalin at 4 °C.

Immunohistochemistry was performed on 4- μ m paraffin sections. The antibodies 029-1, 029-2 (1:250–500), D3E10 (against A β 42, 1:1000; Cell Signaling Technology, Danvers, MA, USA), and IC16 (against the N-terminus of A β , 1:1000) were used for A β staining. Biotinylated secondary anti-guinea pig and anti-mouse antibodies (1:200) were purchased from Dianova and Dako (Glostrup, Denmark), respectively. Staining was visualized using the avidin-biotin complex method with a VECTASTAIN kit (Vector Laboratories) and diaminobenzidine (DAB) as the chromogen. Counterstaining was carried out with hematoxylin.

The A β _{4-x} plaque load was quantified using 9-month-old hemi- and homozygous 5XFAD mice ($n = 3-4$) [28]. In brief, three paraffin sections per animal were stained simultaneously with DAB. The relative A β _{4-x} load in thalamus and cortex was evaluated using a BX51 microscope (Olympus, Center Valley, PA, USA) equipped with a Moticam Pro 282 camera (Motic, Wetzlar, Germany) and ImageJ1.47b software (<http://imagej.nih.gov/ij>). Representative images were captured and binarized to 8-bit black-and-white images using a fixed intensity threshold. Measurements were performed with analysis of the percentage of the area covered by DAB as previously described [27]. Double-immunofluorescence staining was visualized using Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA) and 4',6-diamidino-2-phenylindole using a Nikon C2+ confocal microscope equipped with 405 nm, 488-nm and 561-nm lasers (Nikon Instruments, Melville, NY, USA).

Enzyme-linked immunosorbent assay

The levels of A β ₄₋₄₀ peptides in 5-month-old heterozygous ($n = 5$) and homozygous ($n = 6$) 5XFAD mice were determined using a sandwich enzyme-linked immunosorbent assay (ELISA). Frozen brain hemispheres were weighed and sequentially extracted. First, brains were homogenized in 700 μ l of Tris-buffered saline (120 mM NaCl, 50 mM Tris, pH 8.0, with cOmplete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) per 100 mg of tissue using a Dounce homogenizer (800 rpm). The resulting solution was centrifuged at 17,000 $\times g$ for 20 minutes at 4 °C. The pellet was dissolved in 800 μ l of 2% SDS and sonicated, followed by a centrifugation step of 17,000 $\times g$ for 20 minutes at 4 °C. The supernatant containing SDS-soluble proteins was incubated while rotating with 1 μ l of benzonase at RT for 10 minutes, followed by storage at -80 °C. The SDS brain fraction was used for ELISA analysis. To generate standard curves, synthetic A β ₄₋₄₀ peptides (PSL) were used. A C-terminal-specific antibody for A β ₄₀ (BAP-24) [29] served as a capture antibody and was incubated at 3 μ g/ml in PBS, pH 7.2, on 96-well high-binding

microtiter plates (Greiner Bio-One, Kremsmünster, Austria) overnight at 4 °C. After excess capture antibody was removed, freshly diluted brain samples (1:30) or A β peptide standards (31.25–750 pg/ml) in PBS containing 0.05% Tween 20 and 1% bovine serum albumin (BSA) were added. Then, the A β _{4-x}-specific 029-2 antibody labeled with HRP using the Pierce EZ-Link™ Plus Activated Peroxidase kit (Thermo Fisher Scientific) was diluted in PBS containing 0.05% Tween 20 and 1% BSA, added to each well, and incubated overnight at 4 °C. On the next day, plates were washed three times with PBS containing 0.05% Tween 20 and once with PBS. Subsequently, 50 μ l of trimethylbenzidine ELISA peroxidase substrate (Interchim, Montluçon, France) was added and incubated for 1–5 minutes at RT in the dark. The reaction was terminated by adding 50 μ l of 2 M H₂SO₄, and the absorbance was recorded using a Paradigm microplate reader (Beckman Coulter, Brea, CA, USA) at 450 nm.

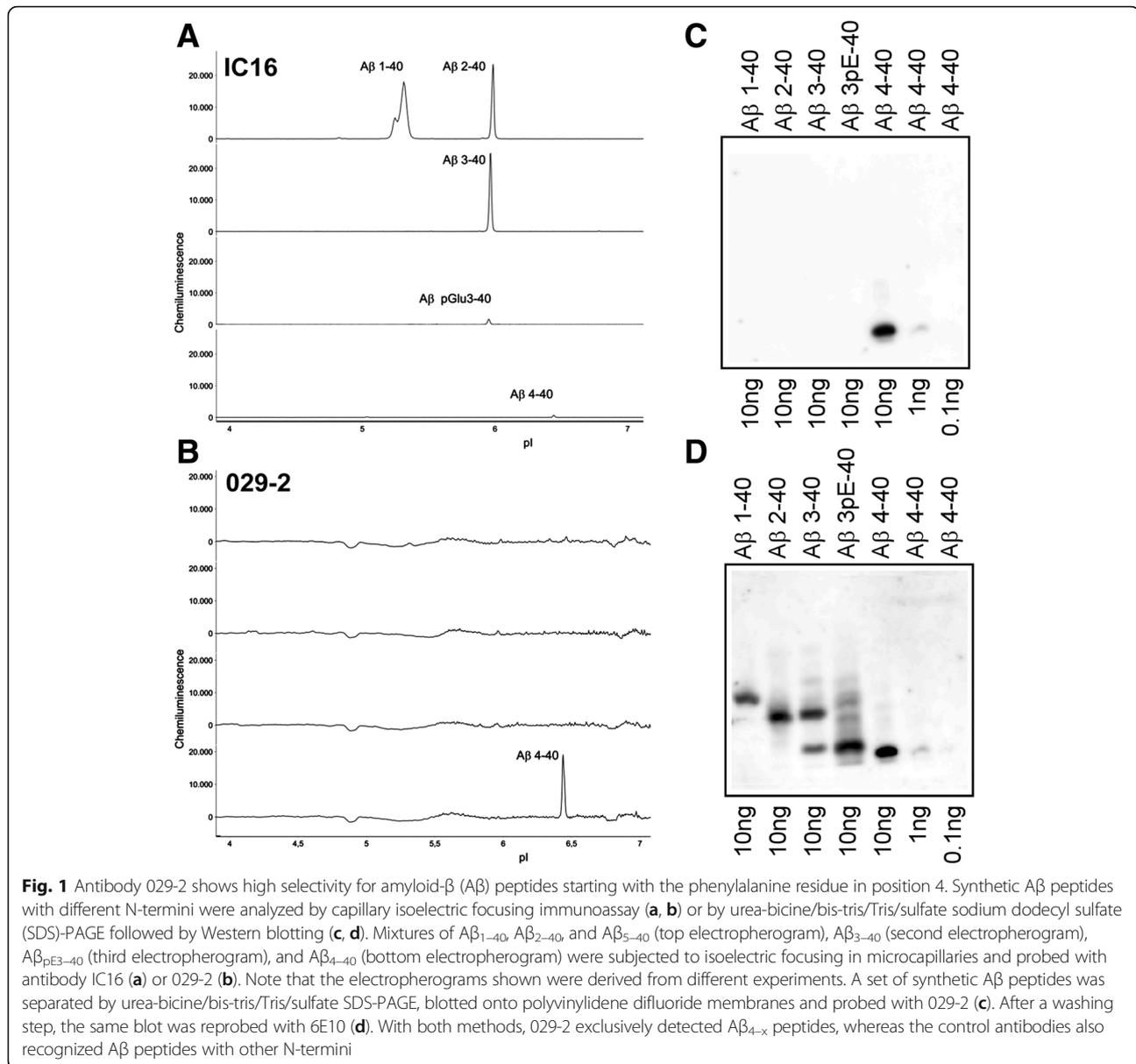
Statistical analysis

Differences between groups were tested by unpaired *t* tests. All data were expressed as mean \pm SD. Significance levels were as follows: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. All calculations were performed using Prism version 6.07 for Windows software (GraphPad Software, La Jolla, CA, USA).

Results

Purified polyclonal antibodies 029-1 and 029-2 show high selectivity for N-truncated A β _{4-x} peptides

The selectivity of the affinity-purified polyclonal antibodies 029-1 and 029-2 for different N-terminal variants of A β was studied by three independent methods. First, a CIEF immunoassay demonstrated that both antibodies detected synthetic A β peptides starting with the Phe residue in position 4 without any noticeable cross-reactivity to other tested A β peptides (Fig. 1b and Additional file 1). In contrast, control antibody IC16 detected the N-terminus of A β with clear signals for A β ₁₋₄₀, A β ₂₋₄₀, and A β ₃₋₄₀. Reactivity to A β _{pE3-40} and A β ₄₋₄₀ was very low, whereas A β ₅₋₄₀ was not detected (Fig. 1a). Antibody 6E10 served as an additional control and detected all synthetic peptides applied (Additional file 1: Figure S1) as previously shown [7]. Second, urea bicine/bis-tris/Tris/sulfate SDS-PAGE followed by Western immunoblotting was performed for 029-2 and indicated that this antibody selectively recognized A β peptides starting with Phe in position 4 in amounts as low as 1 ng. Again, no cross-reactivity was detected against any of the other synthetic A β peptides tested, namely A β ₁₋₄₀, A β ₂₋₄₀, A β ₃₋₄₀, and A β _{pE3-40} (Fig. 1c). Reprobing of the membrane with antibody 6E10 allowed the detection of all tested A β peptides (Fig. 1d). Third, importantly,



preabsorption of 029-2 with synthetic A β ₄₋₄₀ peptides completely blocked 029-2 immunoreactivity in formalin-fixed, paraffin-embedded brain tissue from 5XFAD mice. In contrast, preabsorption with full-length A β ₁₋₄₀ peptides did not result in reduced immunoreactivity, and A β _{4-x}-positive extracellular deposits were still detected (Fig. 2).

A β _{4-x} in sporadic AD cases

On tissue slides from human patients with sporadic AD, 029-2 labeled extracellular amyloid deposits as well as blood vessels (Fig. 3). In contrast to IC16, which detects full-length A β peptides and N-truncated variants and decorates either cored plaques or more diffuse extracellular A β

deposits (Fig. 3a, c, e), 029-2 staining was restricted mainly to compact neuritic plaques (Fig. 3f) and plaque cores (Additional file 2) as well as vascular deposits (Fig. 3b, d). Table 1 summarizes a semiquantitative analysis of 029-2 versus IC16 staining in sporadic AD and nondemented control specimens, in which the immunoreactivity in the form of cerebral amyloid angiopathy (CAA) and extracellular amyloid deposition was assessed. Double-labeling and confocal microscopy using IC16 (red) and 029-2 (green) confirmed the presence of A β _{4-x} peptides predominantly in cored neuritic plaques, whereas more diffuse extracellular deposits showed only IC16 immunoreactivity (Fig. 4a). Confocal analysis of blood vessels revealed partial colocalization of A β _{4-x} peptides with IC16 immunoreactivity in the

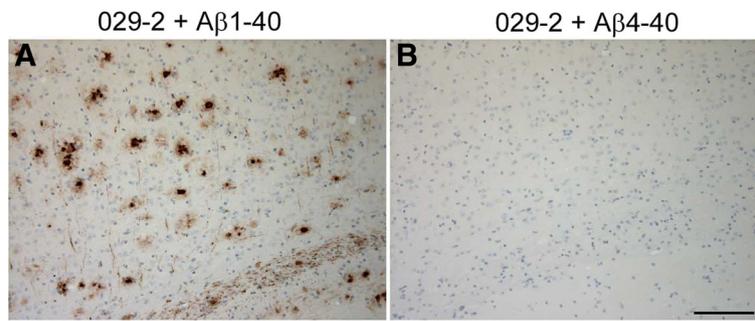


Fig. 2 Whereas preabsorption with synthetic amyloid- β ($A\beta$)₁₋₄₀ did not compromise 029-2 staining in aged 5XFAD mice (a), preabsorption with $A\beta$ ₄₋₄₀ completely abolished 029-2 immunoreactivity (b), underscoring the specificity of the 029-2 antibody for $A\beta$ _{4-x} peptides. Scale bar = 100 μ m

vessel wall. However, in a minor subset of vessels, the vessel walls showed either IC16 or 029-2 immunoreactivity and a nonoverlapping staining pattern (Fig. 4b, arrowheads).

$A\beta$ _{4-x} in transgenic mouse models

With antibody 029-2 against $A\beta$ _{4-x}, abundant extracellular amyloid plaques were detectable in 10-month-old 5XFAD

(Fig. 5b) and 8-month-old APP/PS1KI mice (Fig. 5j). Compared with staining with the $A\beta$ ₄₂-selective antibody D3E10 (Fig. 5a), 029-2 immunoreactivity was restricted to the plaque core (Fig. 5b) in aged 5XFAD mice. Double-labeling with an APP antibody (red) confirmed the localization of $A\beta$ _{4-x} (green) in the plaque core, whereas APP was detected largely within dystrophic neurites in the

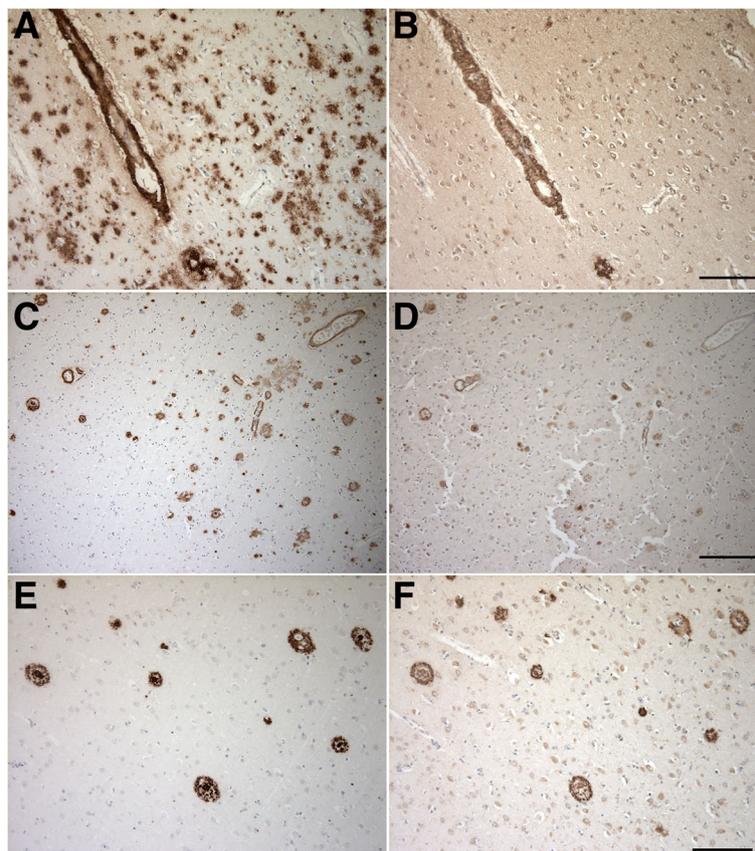


Fig. 3 Immunohistochemical staining of amyloid- β ($A\beta$)_{4-x} peptides in sporadic Alzheimer's disease (AD) cases. With IC16 recognizing full-length $A\beta$ peptides, abundant extracellular (a, c, e) and vascular (a, c) amyloid deposits were detected in sporadic AD brains (a). In contrast, after staining with the $A\beta$ _{4-x} specific 029-2 antibody, immunoreactivity was restricted to blood vessels (b, d) and neuritic plaques (d, f), while diffuse amyloid deposits were completely negative (b). Scale bar = 100 μ m (a, b, e, f); 200 μ m (c, d)

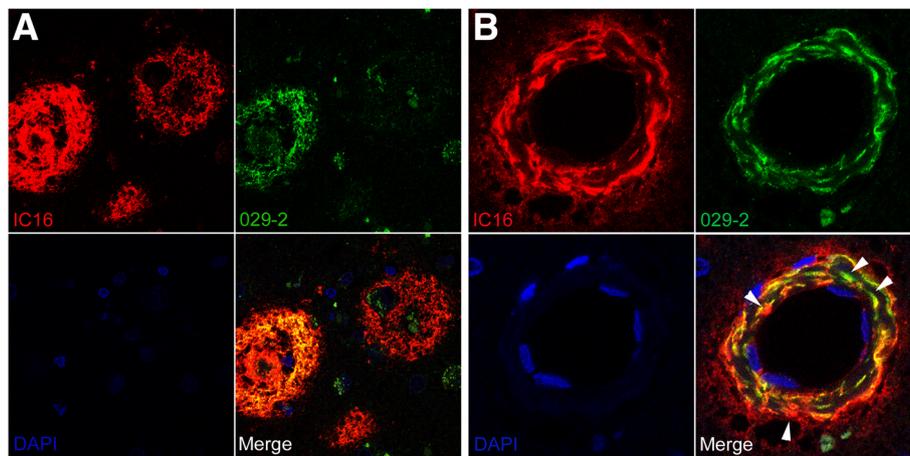


Fig. 4 Fluorescent immunostaining in sporadic Alzheimer's disease cases. Confocal microscopy using IC16 (red), 029-2 (green), and 4',6-diamidino-2-phenylindole (DAPI; blue) reveals colocalization of IC16 and 029-2 staining in cored but not diffuse amyloid deposits (a). In blood vessels, partially overlapping staining profiles were observed; however, distinct immunoreactivity of IC16 and 029-2 also was evident within the vessel wall (b, arrowheads). Scale bar = 33 μ m (a); 15 μ m (b)

vicinity of the amyloid plaque (Fig. 5c–e). This was further supported by double-labeling with D3E10 and 029-2, clearly indicating that $A\beta_{4-x}$ immunoreactivity was restricted to the central plaque core (Fig. 5f–h). Similar results were obtained for the APP/PS1KI line, in which D3E10 displayed a more diffuse and dissipated staining pattern (Fig. 5i), whereas 029-2 labeled only the central plaque core (Fig. 5j). A quantitative analysis of extracellular $A\beta_{4-x}$ -positive deposits using antibodies 029-1 and 029-2 was carried out in the cortex, subiculum, and thalamus of 9-month-old heterozygous and homozygous 5XFAD mice (Fig. 6a and Additional file 3). For both antibodies, a significantly increased plaque load could be detected in all analyzed areas in homozygous compared with heterozygous 5XFAD mice (all $p < 0.05$). In addition, to evaluate the suitability of the 029-2 antibody for ELISA quantification, $A\beta_{4-40}$ levels were determined in the SDS-soluble brain fraction of 5-month-old heterozygous and homozygous 5XFAD mice. As expected, significantly increased $A\beta_{4-40}$ levels were detected in homozygous 5XFAD mice ($p < 0.001$), supporting a gene dose-dependent increase in insoluble $A\beta_{4-40}$ peptide levels (Fig. 6b).

Discussion

In addition to full-length $A\beta$ peptides starting with an aspartate in position 1 ($A\beta_{1-x}$), a variety of N-terminally truncated and posttranslationally modified $A\beta$ peptides have been detected in human AD brains [14, 16, 17]. N-terminal truncations were shown to promote the aggregation propensity of $A\beta$ peptides [18]. However, the presence of N-truncated peptides has been demonstrated mainly by mass spectrometry following immunoprecipitation with generic $A\beta$ antibodies such as 4G8 or

6E10 [14, 15, 21], and their genesis in vivo is mostly unresolved. Consequently, the functional role of N-truncated $A\beta$ peptides in the pathogenesis of AD has remained unclear, including fundamental questions about the abundance and distribution of N-truncated isoforms. This shortcoming has been particularly obvious for the $A\beta_{4-x}$ peptides starting with Phe in position 4, which have been proposed to be an abundant $A\beta$ species in AD brains but for which no specific antibodies have been generated to date. We have previously reported a monoclonal antibody, NT4X-167, that preferentially detected $A\beta_{4-x}$ peptides and protected primary cortical neurons from the toxicity of $A\beta_{4-42}$ peptides in vitro [30]. However, in addition to monomeric and oligomeric $A\beta_{4-x}$ peptides, NT4X-167 was shown to recognize $A\beta_{pE3-x}$ peptides. Hence, this antibody is not suitable for accurate measurement of the abundance and distribution of $A\beta_{4-x}$ peptides. We have now raised polyclonal antibodies by immunizing guinea pigs with the six-amino acid peptide (FRHDSG) corresponding to residues 4–9 of the $A\beta$ peptide sequence. The specificity of these antibodies for $A\beta_{4-x}$ peptides was confirmed by CIEF immunoassay and urea SDS-PAGE, and no cross-reactivity for $A\beta_{1-40}$, $A\beta_{2-40}$, $A\beta_{3-40}$, $A\beta_{pE3-40}$, and $A\beta_{5-40}$ was observed. Furthermore, in immunohistochemical staining, the immunoreactivity could be entirely blocked by preabsorption with $A\beta_{4-40}$ but not $A\beta_{1-40}$ peptides. Two independent animals were immunized and yielded two antisera, 029-1 and 029-2, with nearly identical immunoreactivity, indicating that $A\beta_{4-9}$ might be a reliable immunogen to raise $A\beta_{4-x}$ -specific antibodies in guinea pigs. Compared with the well-established antibody IC16, which preferentially detects full-length $A\beta$ peptides, the immunohistochemical staining patterns of the newly

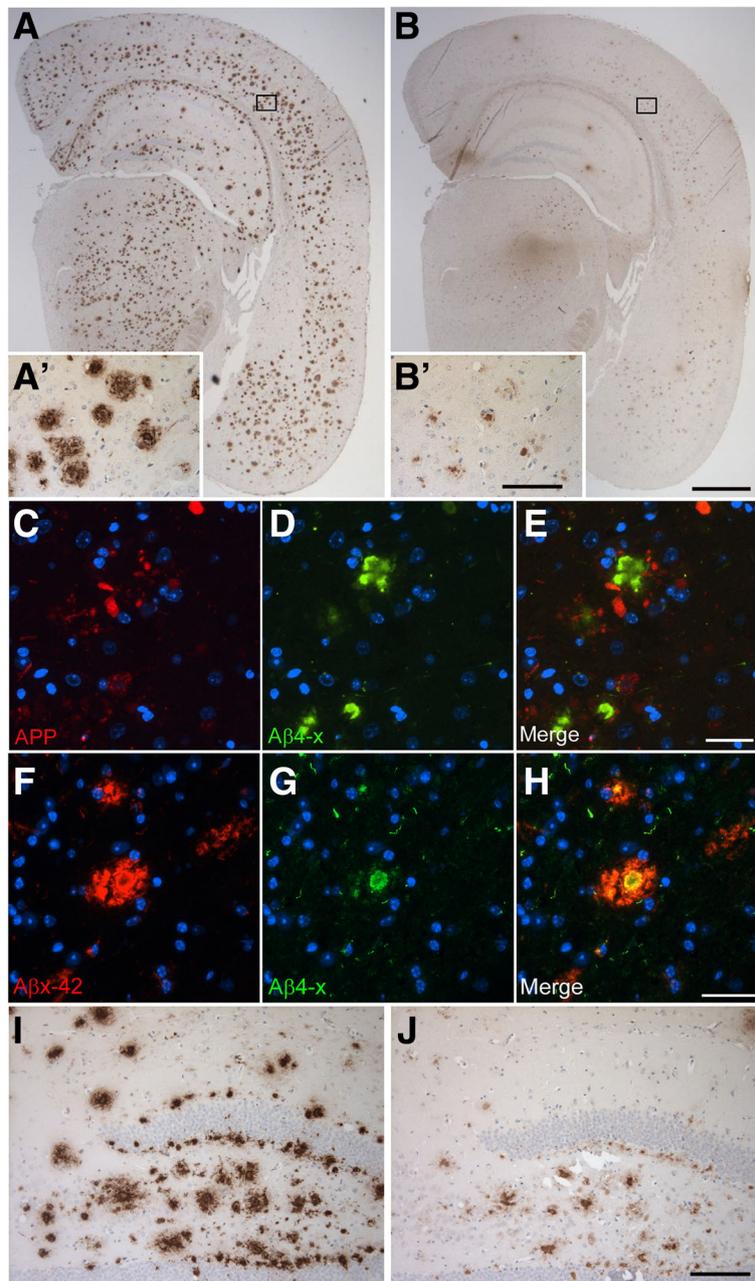


Fig. 5 Amyloid- β ($A\beta$)_{4-x} peptides in transgenic Alzheimer's disease (AD) mouse models. Using antibody D3E10 against $A\beta$ ₄₂, abundant extracellular deposits were detected in 10-month-old 5XFAD mice by immunohistochemistry (**a, a'**). In contrast, the $A\beta$ _{4-x}-specific antibody 029-2 showed a strongly reduced staining pattern that was restricted to amyloid cores (**b, b'**). Staining with an amyloid precursor protein (APP) antibody decorated only dystrophic neurites (**c**) surrounding the $A\beta$ _{4-x}-positive plaque core (**d, e**). The restricted $A\beta$ _{4-x} immunoreactivity within plaque cores was further demonstrated using double-labeling of D3E10 (**f**) and 029-2, which decorated only the central plaque core (**g, h**). A similar staining pattern to that in 5XFAD mice was observed in 8-month-old APP/PS1KI mice, as exemplified in the hippocampus (D3E10 [**i**] and 029-2 [**j**]). Scale bar = 1000 μ m (**a, b**); 60 μ m (**a', b', f-h**); 33 μ m (**c-e**); 100 μ m (**i, j**)

generated $A\beta$ _{4-x}-specific antibodies were quantitatively and qualitatively different. In brain sections of both patients with sporadic AD and two AD mouse models, the distribution of $A\beta$ _{4-x} peptides was restricted largely to amyloid plaque cores and CAA, whereas diffuse amyloid

deposits were negative. The presence of $A\beta$ _{4-x} peptides in amyloid plaque cores raises the question whether these truncated species are critical in the very early stages of the pathology. We have not yet conducted a comprehensive longitudinal study comparing different

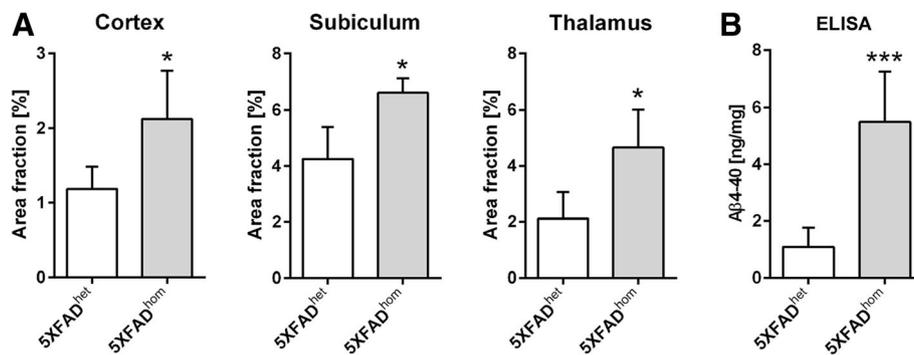


Fig. 6 Increased amyloid- β ($A\beta_{4-x}$) levels in homozygous 5XFAD mice **a**. Quantification of extracellular 029-2-positive plaque load in cortex, subiculum, and thalamus in heterozygous and homozygous 9-month-old 5XFAD mice. **b** Quantification of sodium dodecyl sulfate-soluble $A\beta_{4-40}$ levels in brains of heterozygous and homozygous 5-month-old 5XFAD mice revealed a significant gene dose-dependent increase. *** $p < 0.001$; * $p < 0.05$. ELISA Enzyme-linked immunosorbent assay, FAD Familial Alzheimer's disease

animal ages and time points before or after the onset of amyloid deposition. However, using two-dimensional Western blotting combined with mass spectrometry, N-terminally truncated $A\beta$ peptide species starting at position 4 or 5 have already been detected at 2.5 months of age in the APP/PS1KI line, indeed indicating a very early appearance of these truncated species [26]. In good agreement and using a similar experimental approach, Sergeant and colleagues reported that $A\beta$ aggregates at the first stages of amyloid deposition in nondemented individuals with amyloid and tau pathologies are composed predominantly of N-truncated variants, including $A\beta_{4-x}$ peptides [31]. Antibodies raised in guinea pigs are especially useful for colocalization studies because most high-quality antibodies against other $A\beta$ species or APP fragments have previously been generated in either mice or rabbits. Indeed, double-immunofluorescence staining demonstrated $A\beta_{4-x}$ -positive amyloid plaque cores decorated by APP-positive dystrophic neurites with no overlap in the fluorescent signals. In line with this observation, no intraneuronal staining was observed for $A\beta_{4-x}$ peptides in mice at the ages of 8–10 months. However, it could be worth studying younger animals because intraneuronal $A\beta$ accumulation is most prominent in young mice prior to amyloid plaque formation [32]. Overall, the distribution of $A\beta_{4-x}$ peptides was also substantially different from the staining pattern reported for other N-truncated species, including $A\beta_{2-x}$ [7] or $A\beta_{5-x}$ [10, 11], which were not or less confined to cored neuritic plaques. Previous studies have demonstrated that $A\beta_{4-x}$ peptides rapidly formed soluble oligomers and fibrillar higher-molecular-weight aggregates [33]. This biochemical property might explain not only the confined localization of $A\beta_{4-x}$ peptides to amyloid cores but also their high neurotoxicity in vitro and in vivo. Short-term exposure of primary cortical neuron cultures to $A\beta_{4-40}$ and $A\beta_{4-42}$ peptides resulted in a

concentration-dependent cytotoxic effect with comparable effect sizes to $A\beta_{1-42}$. Furthermore, the expression of $A\beta_{4-42}$ under the control of a neuronal promoter caused age-dependent behavioral deficits and hippocampal neuron loss in a transgenic mouse model (Tg4-42) [33].

Another important unresolved issue is the abundance of $A\beta_{4-x}$ peptides in relation to full-length $A\beta$ peptides in both AD and transgenic mouse models of the disease. To start to address this issue and to evaluate the novel $A\beta_{4-x}$ antibodies for quantitative analysis, we combined the 029-2 antibody with a C-terminus-specific $A\beta_{40}$ antibody [29] in a sandwich ELISA. In the SDS-soluble brain fraction of 5-month-old heterozygous 5XFAD mice, this assay detected around 1 ng of $A\beta_{4-40}$ per milligram of tissue with approximately fivefold higher levels in homozygous 5XFAD mice of the same age. In a previous study, we had determined the levels of full-length $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides in the same brain extracts of the same animal cohort with a comparable ELISA system and IC16 as a capture antibody [28]. Combining the results from both studies indicates that $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides are approximately 75-fold and 200-fold more abundant, respectively, than $A\beta_{4-40}$ peptides in the 5XFAD mouse model at 5 months of age. This also fits with a matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis of formic acid brain extracts of 7-month-old 5XFAD mice. Although peptide concentrations cannot be deduced from peak heights in mass spectra, this analysis showed that the signal intensities generated by the $A\beta_{4-40}$ and $A\beta_{4-42}$ peptides were only a small fraction of the $A\beta_{1-40}$ and $A\beta_{1-42}$ peaks [5]. Taken together, these results indicate that, quantitatively, N-truncated $A\beta_{4-x}$ peptides are a minor $A\beta$ species in the 5XFAD mouse model. However, evidence from human studies suggests that the proportions of N-truncated to full-length $A\beta$ peptides might

be substantially different in the brains of patients with AD. Already more than 30 years ago, it was shown by N-terminal sequencing analysis of A β peptides purified from amyloid plaque cores that only around 10% of peptides displayed an intact N-terminus, whereas > 60% started with the Phe residue in position 4 [19]. Another sequencing study confirmed that A β peptides starting with Phe represented the major component of plaques, whereas full-length A β starting with Asp was detected predominantly in the vasculature [20]. In addition, later studies using mass spectrometry have generally supported that A β_{1-42} , A β_{pE3-42} , and A β_{4-42} belong to the A β peptide species with a high prevalence in AD brains [14, 15, 21, 34]. Finally, comparative biochemical studies of amyloid plaques isolated from human AD brains and APP transgenic mouse models have also shown that N-truncated A β peptides are much more prevalent in patients with AD, and it has been proposed that the greater abundance of N-truncated A β peptides is at least in part responsible for the substantially lower solubility of amyloid plaque material from humans [35, 36]. In any case, additional studies in human AD brains using genuinely quantitative methods to determine the abundance of A β_{4-x} peptides and their distribution in soluble and insoluble brain fractions are clearly warranted. How A β_{4-x} peptides are generated remains entirely unclear. Although there is evidence that enzymatic activities can facilitate N-terminal A β truncations such as A β_{2-x} [37, 38], no enzymes have yet been identified that are able to generate A β_{4-x} peptides. Nonenzymatic generation of truncated A β peptides has also been proposed, and it has been shown that full-length A β peptides can spontaneously decompose into shorter N- and C-terminally truncated isoforms in vitro in the absence of proteases [39, 40]. With respect to N-terminal truncations, spontaneous decomposition of full-length to A β_{3-x} peptides with subsequent cyclization of the N-terminal Glu to pGlu has been demonstrated, but there is only very limited evidence that a similar process could produce A β_{4-x} peptides [39]. Our finding that A β_{4-x} peptides are confined largely to amyloid cores supports an important role of these N-truncated A β species in the process of amyloid plaque formation. Beyond that, further understanding of the pathological relevance of A β_{4-x} peptides will likely require clarification of their origin and the subsequent generation of genetic loss-of-function models.

Conclusions

Antibodies developed in the present study selectively detect A β_{4-x} species and might represent useful research tools for immunohistochemical or biochemical analyses

of the occurrence or distribution of these peptides. Our analysis reveals that A β_{4-x} is restricted mainly to amyloid plaque cores and CAA in AD and DS cases, as well as in the 5XFAD and APP/PS1KI transgenic mouse lines of AD. This is in line with previous reports demonstrating the high aggregation propensity of these N-terminally truncated A β variants.

Additional files

Additional file 1: Figure S1. CIEF electropherograms for antibodies 6E10 and 029-1. (PDF 184 kb)

Additional file 2: Figure S2. Quantification of extracellular 029-1 positive plaque load in cortex, subiculum, and thalamus in heterozygous and homozygous 9-month-old 5XFAD mice. (PDF 119 kb)

Additional file 3: Figure S3. Predominant plaque core staining using 029-2 in a patient with sporadic AD. (PDF 183 kb)

Abbreviations

A β : Amyloid- β ; AD: Alzheimer's disease; ApoE: Apolipoprotein E; APP: Amyloid precursor protein; BSA: Bovine serum albumin; CAA: Cerebral amyloid angiopathy; CIEF: Capillary isoelectric focusing; DAB: Diaminobenzidine; DAPI: 4',6-Diamidino-2-phenylindole; DMSO: Dimethyl sulfoxide; DS: Down syndrome; ECL: Electrochemiluminescence; ELISA: Enzyme-linked immunosorbent assay; FAD: Familial Alzheimer's disease; IgG: Immunoglobulin G; KLH: Keyhole limpet hemocyanin; NDC: Nondemented control subject; PBS-T: Phosphate-buffered saline with 0.1% Tween 20; Phe: Phenylalanine; PSEN1: Presenilin-1; PSL: Peptide Specialty Laboratory; RT: Room temperature; SDS: Sodium dodecyl sulfate; SP: Senile plaques

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Authors' contributions

OW designed the study, performed experiments, and together with SWe drafted the manuscript and analyzed the data. SWa, IK, HWK, MS and TJO performed experiments and contributed to revision of the manuscript. JG, TAB, and JW provided reagents and contributed to the interpretation of findings and revision of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures involving human tissue were approved by the ethics committee of the University Medical Center Göttingen (protocol number 12/1/15). All tissues were received from the Netherlands Brain Bank (NBB), and all materials and data collected by the NBB are obtained on the basis of written informed consent. All experimental procedures in the study that involved animals were approved by the institutional animal care and use committee at University Medicine Göttingen.

Consent for publication

All authors gave consent for publication.

Competing interests

The authors declare that they have no competing interests.

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