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Abundance of A β_{5-x} like immunoreactivity in transgenic 5XFAD, APP/PS1KI and 3xTG mice, sporadic and familial Alzheimer's disease

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Abstract

Background: According to the modified amyloid hypothesis the main event in the pathogenesis of Alzheimer's disease (AD) is the deposition of neurotoxic amyloid β -peptide (A β) within neurons. Additionally to full-length peptides, a great diversity of N-truncated A β variants is derived from the larger amyloid precursor protein (APP). Vast evidence suggests that A β_{x-42} isoforms play an important role triggering neurodegeneration due to its high abundance, amyloidogenic propensity and toxicity. Although N-truncated and A β_{x-42} species have been pointed as crucial players in AD etiology, the A β_{5-x} isoforms have not received much attention.

Results: The present study is the first to show immunohistochemical evidence of A β_{5-x} in familial cases of AD (FAD) and its distribution in APP/PS1KI, 5XFAD and 3xTG transgenic mouse models. In order to probe A β_{5-x} peptides we generated the AB5-3 antibody. Positive plaques and congophilic amyloid angiopathy (CAA) were observed among all the FAD cases tested carrying either APP or presenilin 1 (PS1) mutations and most of the sporadic cases of AD (SAD). Different patterns of A β_{5-x} distribution were found in the mouse models carrying different combinations of autosomal mutations in the APP, PS1 and Tau genes. All of them showed extracellular A β deposits but none CAA. Additionally, they were all affected by a severe amyloid pathology in the hippocampus among other areas. Interestingly, neither 5XFAD nor APP/PS1KI showed any evidence for intraneuronal A β_{5-x} .

Conclusions: Different degrees of A β_{5-x} accumulations can be found in the transgenic AD mouse models and human cases expressing the sporadic or the familial form of the disease. Due to the lack of intracellular A β_{5-x} , these isoforms might not be contributing to early mechanisms in the cascade of events triggering AD pathology. Brain sections obtained from SAD cases showed higher A β_{5-x} -immunoreactivity in vascular deposits than in extracellular plaques, while both are equally important in the FAD cases. The difference may rely on alternative mechanisms involving A β_{5-x} peptides and operating in a divergent way in the late and early onset forms of the disease.

Keywords: A β_{5-x} , Amyloid plaques, 5XFAD, 3xTG, APP/PS1KI, Vascular deposits, N-truncated A β

Background

Alzheimer's disease (AD) is the most common type of dementia worldwide. It is characterized by the accumulation of specific proteins, namely tau and amyloid-beta protein (A β). In fact, these proteins are essential to confirm an AD diagnosis, given that the two major histopathological hallmarks are extracellular amyloid- β plaques surrounded by

dystrophic neurites and intracellular neurofibrillary tangles. Furthermore, AD is characterized by neuronal loss, gliosis and congophilic angiopathy mainly affecting the cortex and the hippocampal formation [1].

The amyloid hypothesis considers the accumulation of A β peptides as the central and triggering event in AD [2,3]. The formation of neurotoxic oligomers and larger assemblies of A β are thought to be the product of an imbalance in its production and clearance [4]. A β derives from the larger amyloid precursor protein (APP) by proteolytic cleavage of different secretase enzymes. The

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combined activity of β - and γ -secretase activities releases A β peptides of various lengths [5]. The γ -secretase comprises a high molecular weight complex that depends on presenilin-1 and -2 (PS1, PS2) activity to cleave within the transmembrane domain of APP to generate A β peptides and is composed of four integral membrane proteins: presenilin, nicastrin, Aph-1 and Pen-2 [6]. Supporting the amyloid hypothesis, autosomal dominant mutations in APP, PS1 and PS2 genes cause familial early onset AD mainly by increasing the production of A β_{x-42} [7]. However, advancing in age is considered the most prevalent risk factor for A β accumulation and most of the cases have a late onset. These cases are classified as sporadic AD.

Extracellular plaques are formed by A β peptides with different C-termini ranging from position 38 to 43 [5]. Since the 42 amino acid isoforms A β_{x-42} are highly susceptible to aggregate and to form oligomer and amyloid fibrils [8], it is considered the main plaque component and the initiator of plaque formation in AD pathogenesis [9]. In addition to the most prevalent species A β_{x-40} and A β_{x-42} , other isoforms such as A β_{x-38} has been reported in different mouse models, FAD cases due to mutations in APP and PS1 and in the vascular A β deposits of SAD cases [10,11]. A β species ending at position 43 have shown to be potentially amyloidogenic and abundant [12]. Additionally to the full length A β peptides starting with N-terminal aspartate at position 1, different N-truncated isoforms have been demonstrated to be as abundant as toxic due to their capacity to rapidly form stable aggregates [13]. Not much is known about the N-terminally truncated A β_{5-x} present in the amyloid pathology of AD brains. It has been suggested that these A β variants are preferentially formed by an alternative cleavage of APP involving caspase activity [14].

The aim of the present work was to characterize our recently generated antibody as a tool to study the presence of A β_{5-x} in different AD transgenic mouse models and human cases, including sporadic AD and familial AD cases carrying either APP or PS1 mutations.

Material and methods

Antibodies

The rabbit polyclonal antibodies AB5-3 and 24311 were generated by PSL Heidelberg (will be distributed by T.A.B. upon request). 24311 recognizes pan-A β (epitope A β_{4-40}) and AB5-3 recognizes the free N-terminus of A β_{5-42} (epitope A β_{5-42}). The polyclonal rabbit antibody A β_{42} was purchased from Synaptic Systems, Goettingen. The monoclonal antibody 4G8 is a pan-A β antibody (epitope 17-24; Covance). All antibodies were affinity purified.

Human brain samples

We examined the brains of 12 sporadic AD and 9 non-demented control patients (Table 1). In addition, we

studied brains from familial AD patients either carrying APP or PS1 mutations. The APP mutations KM670/671NL (Swedish) [15] and E693G (Arctic) [16,17] were included as well as three different subjects carrying the PS1 delta exon 9 mutation [18]. Human brain samples were obtained from the Netherlands Brain Bank (NBB), Hopital de la Salpetriere (a generous gift of C. Duyckaerts and V. Sazdovitch), University Hospital Helsinki and from Uppsala University. Definite diagnosis was based on established criteria and written informed consent was obtained from all subjects.

Transgenic mouse brain samples

Brain sections were obtained from 3 established AD transgenic mouse models including 3xTg [19], APP/PS1KI [20] (generous gift by Dr. Laurent Pradier, Sanofi) and 5XFAD [21]. The 5XFAD mice have been crossed on C57Bl6 background [22].

Sample preparation

The amyloid β (A β) variants A β_{1-40} , A β_{1-42} , A β_{4-38} , A β_{4-40} , A β_{4-40} , A β_{5-42} , A β_{pE3-40} , and A β_{pE3-42} were purchased from Peptide Specialty Laboratory (PSL, Heidelberg) and used without further purification as previously described [23]. Briefly, A β peptides were dissolved in 10 mM NaOH at a concentration of 1 mg/mL and aliquoted in 50 μ l volumes. After flash freezing in liquid nitrogen, the aliquots were stored at -80°C until use.

Analysis of AB5-3 using dot blotting

2 μ l of different A β isoforms were spotted on a nitrocellulose membrane in different quantities (2, 1, 0.5, 0.25 and 0.125 μ g). The membrane was dried for 20 minutes and blocked with 10% non-fat dry milk in TBS-T (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 30 minutes. Primary antibodies AB5-3 (diluted 1:500) and 24311 (pan-A β , 1:500) were incubated overnight at 4°C . Running and transfer buffers were applied according to the manufacturer. The blots were developed using enhanced chemiluminescence according to the manufacturer (Roth). Horse radish conjugated swine anti-rabbit antibody was used as a secondary antibody (1:3,000, Dianova).

Electrophoresis and Western blotting of synthetic peptides

For Western blot analysis under reducing conditions, 2 μ g of each A β peptide was loaded per lane after heating at 95°C for 5 minutes on 4 - 12% Tris-Tricin VarioGels (Anamed), transferred to 0.45 μ m nitrocellulose membranes (GE Healthcare) and detected using the primary antibodies AB5-3 (diluted 1:500) and 4G8 (diluted 1:2000). Running and transfer buffers were applied according to the manufacturer. The blots were developed using enhanced chemiluminescence according to the manufacturer (Roth).

Table 1 Demographic data and semiquantitative analysis of antibody stainings in SAD, FAD and non-demented control cases

Case	Age	Gender	Braak stage	A β _{5-x}		A β	
				SP	CAA	SP	CAA
Sporadic AD cases:							
AD-1	88	F	IV	+	-	+++	++
AD-2	84	F	IV	+	-	+++	-
AD-3	86	M	IV	+	++	++	+++
AD-4	88	F	IV	+	-	++	-
AD-5	84	F	IV	+	++	+++	++
AD-6	93	M	IV	-	+	++	+++
AD-7	92	M	IV	-	+	+++	++
AD-8	92	F	IV	+	++	+++	+++
AD-9	91	F	IV	+	++	+++	+++
AD-10	91	M	IV	+	+	++	+++
AD-11	92	F	IV	-	+	++	+++
AD-12	91	F	IV	-	-	+++	++
Non-demented control cases:							
NDC-1	70	M	0	-	-	-	-
NDC-2	90	F	I	+	+	++	++
NDC-3	88	F	I	+	-	++	-
NDC-4	73	M	0	-	-	-	-
NDC-5	91	M	I	-	-	-	-
NDC-6	78	F	I	+	-	++	-
NDC-7	84	M	I	-	-	-	-
NDC-8	78	M	I	+	-	++	++
NDC-9	82	F	I	-	-	-	-
Familial AD cases							
APPE693G	64	M	n.a.	++	++	+++	+++
APPKM670/671NL	61	F	n.a.	++	++	+++	+++
PS1 Δ Ex9	69	M	n.a.	++	+	+++	+
PS1 Δ Ex9	61	M	n.a.	++	++	+++	+
PS1 Δ Ex9	64	M	n.a.	+	+	+++	+

Abbreviations: SP senile plaques, CAA congophilic amyloid angiopathy.

Immunohistochemistry

Immunohistochemistry was performed on 4 μ m sagittal paraffin sections, as described previously [23]. In brief, sections were deparaffinized in xylene, followed by rehydration in a series of ethanol (70%, 95%, and 100%). After treatment with 0.3% H₂O₂ in 0.01 M PBS to block endogenous peroxidases, sections were boiled in 0.01 M citrate buffer pH 6.0 for antigen retrieval, followed by 3 min incubation in 88% formic acid. Non-specific binding sites were blocked by treatment with skim milk and fetal calf serum in PBS for 1 h at room temperature (RT), prior to the addition of the primary antibodies. Rabbit polyclonal antibodies against A β _{5-x} (1:100, AB5-3) and A β ₄₂ (1:500, Synaptic Systems, Goettingen, Germany), as well as mouse monoclonal 4G8

(1:5000, Covance, Dedham, MA, USA) and the rabbit polyclonal 24311 (1:500, [23]) pan-A β antibodies were incubated overnight in a humid chamber at RT. This was followed by incubation with the corresponding biotinylated secondary antibodies (1:200, DAKO, Glostrup, Denmark) at 37°C before visualization of the staining using the ABC method with a Vectastain kit (Vector Laboratories, Burlingame, USA) and diaminobenzidine (DAB) as chromogen. Counterstaining was carried out with hematoxylin.

Results

Antibody specificity

The specificity of the polyclonal rabbit antibody AB5-3 was tested by dot blot (Figure 1) and western blot analysis

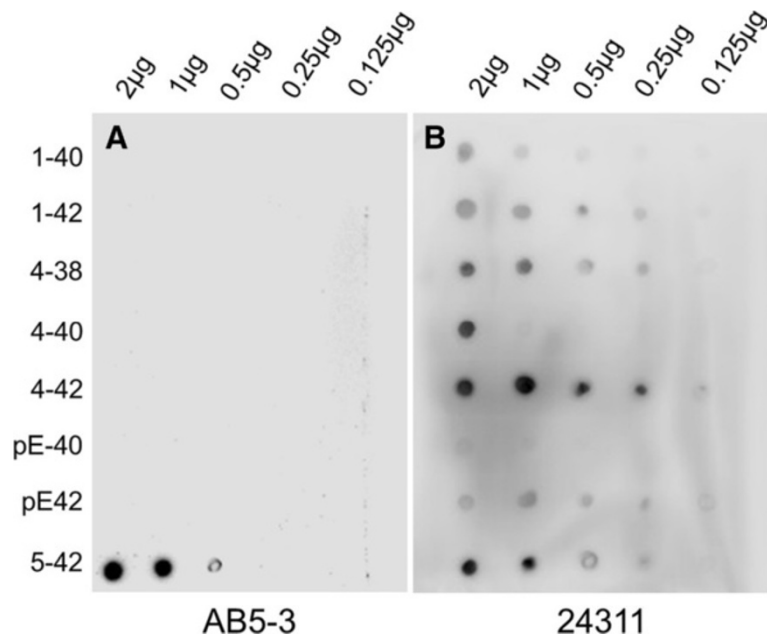


Figure 1 Analysis of the AB5-3 using dot blotting. Full length and N-truncated amyloid- β peptides were probed and spotted into a nitrocellulose membrane. **(A)** After incubation with the primary antibody AB5-3 only the $A\beta_{5-42}$ variant was recognized indicating specificity for this peptide. **(B)** The pan- $A\beta$ antibody 24311 recognized all $A\beta$ variants demonstrating that all peptides were available.

(Figure 2). AB5-3 showed no cross-reactivity with full-length peptides starting with aspartate at position 1 and other N-truncated $A\beta$ isoforms starting with pyroglutamate at position 3 or phenylalanine at position 4. Under denaturing conditions AB5-3 reacted only with $A\beta_{5-42}$, thus detecting ragged peptides starting with arginine at position 5. Data obtained by western blot indicates that

AB5-3 is binding to low molecular weight forms of the peptide detecting only soluble $A\beta_{5-42}$ trimers and tetramers (Figure 2).

$A\beta_{5-x}$ in sporadic Alzheimer's disease

Vascular $A\beta$ deposits of variable degree (Figure 3A, B, E, and F) were detected in two-thirds (8 of 12) of the SAD

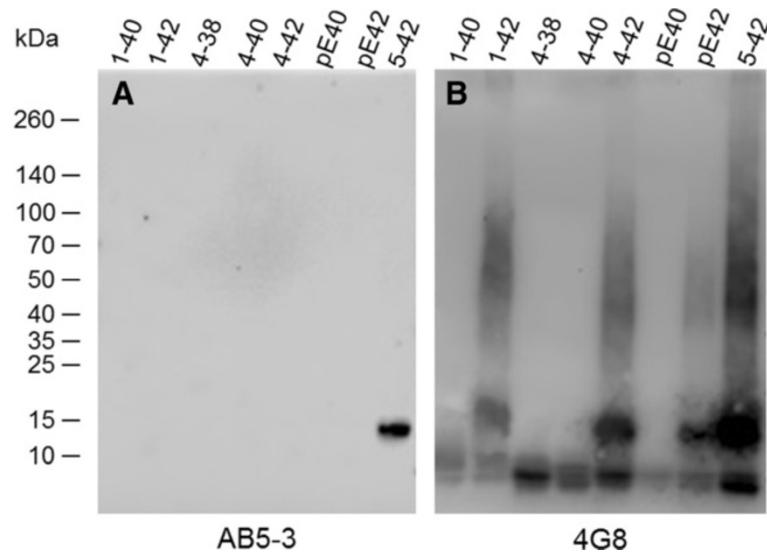


Figure 2 SDS-PAGE Western blot analysis of the AB5-3 and pan- $A\beta$ 4G8 antibodies. Freshly dissolved synthetic $A\beta$ variants (2 µg each) were probed to a membrane. **(A)** The polyclonal AB5-3 antibody detects $A\beta_{5-42}$ trimers and tetramers while **(B)** the monoclonal pan- $A\beta$ antibody 4G8 detects different sizes of all $A\beta$ species.

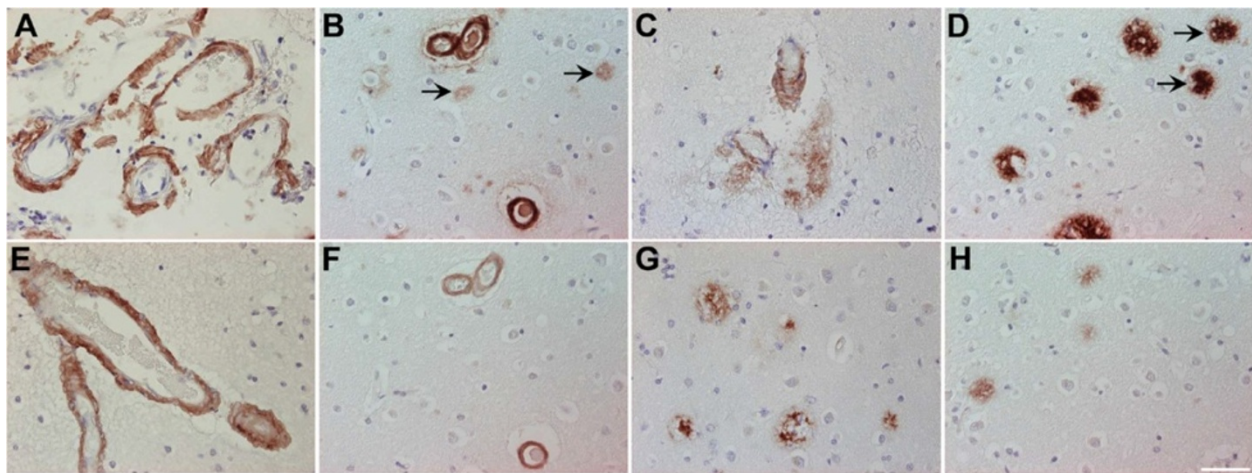


Figure 3 Immunohistochemical staining pattern of $A\beta_{5-x}$ in cortex of sporadic AD human brain. Vascular $A\beta_{5-x}$ immunoreactivity (A, E, F) was detected in the majority of the SAD cases analyzed in hippocampus (A) and cerebral cortex (E), whereas extracellular $A\beta_{5-x}$ -positive deposits (C, G, H) were less abundant in both brain areas (C, D). The pan- $A\beta$ antibody 24311 (B, D) demonstrates cerebral amyloid angiopathy (CAA) and plaque load (arrows). The same cortical region stained in a parallel section with the AB5-3 antibody (F, H) binds preferentially to blood vessels presenting CAA and barely recognizes plaques. Scale bar: 50 μ m.

cases, as well as in one non-demented control case, using immunohistochemical analysis with the AB5-3 antibody. The extent of vascular immunoreactivity corresponded to the severity of CAA, which was assessed using the pan- $A\beta$ antibody 4G8 (Table 1). Extracellular $A\beta_{5-x}$ -immunoreactivity was observed in 8 of 12 SAD cases (Figure 3C, D, G, and H) and in 4 control cases. Although plaque pathology and vascular deposits were present in equal number of SAD cases, vascular immunoreactivity appeared stronger than the parenchymal staining, probably due to a higher abundance of $A\beta_{5-x}$ in vessels (Figure 3A, E and F). Using the 4G8 pan- $A\beta$ antibody, all subjects were proven to harbor a considerable extracellular plaque load with most of them (10 of 12) showing CAA in addition (Table 1). Consequently, extracellular $A\beta_{5-x}$ isoforms were detected in 66% of the subjects affected with plaque pathology and vascular $A\beta_{5-x}$ immunoreactivity in 83% of the subjects affected with CAA. Both diffuse and dense core plaques were positive.

$A\beta_{5-x}$ in familial AD

Samples from five different subjects carrying different autosomal dominant mutations, either in the APP or PS1 gene, were probed with the AB5-3 antibody. All familial cases analyzed in the present study showed prominent vascular and extracellular $A\beta_{5-x}$ -immunoreactivity. Two cases harbored APP pathogenic mutations, either located at codon 693 within the $A\beta$ region of APP (E693G APP) known as the Arctic mutation or a double mutation at codons 670 and 671 close to the β -secretase cleavage site (KM670/671NL APP) known as the Swedish mutation. On one hand, the case carrying the Arctic mutation showed

strong vascular $A\beta_{5-x}$ -immunoreactivity and abundant extracellular plaque deposits. On the other hand, brain sections obtained from the subject carrying the Swedish mutation presented a slightly stronger $A\beta_{5-x}$ -signal in the vasculature and less $A\beta_{5-x}$ -immunoreactivity in plaques (Figure 4). Both cases were compared to adjacent sections stained with the 4G8 pan- $A\beta$ antibody which demonstrated abundant extracellular plaque pathology and CAA.

The cases harboring the PS1 mutation with the exon 9 deletion also showed abundant vascular staining and less pronounced in plaques.

Both diffuse and dense core plaques were positive.

$A\beta_{5-x}$ in mouse models of AD

The present report provides the first immunohistochemical analysis of $A\beta_{5-x}$ in transgenic mouse models of AD. We analyzed three different transgenic lines, namely APP/PS1KI, 5XFAD and 3xTg (Table 2), which express different combinations of human mutant APP, PS1 and Tau. All of the mouse models showed $A\beta_{5-x}$ -positive amyloid plaques to a different extent but none of them revealed $A\beta_{5-x}$ -positive vascular deposits as expected (Figures 5 and 6). Brain sections of the 3xTg mice (18-month-old) showed amyloid deposits localized only in the subiculum of the hippocampus and the medulla (Figure 5E and J). Moreover, abundant $A\beta_{5-x}$ positive plaques were observed in cortex, hippocampus, thalamus and piriform cortex in 10-month-old APP/PS1KI and 12-month-old 5XFAD mice (Figure 5B, E, C and F). Interestingly, the $A\beta_{5-x}$ peptides seemed to be not very abundant and were mostly restricted to the core of plaques (Figure 6).

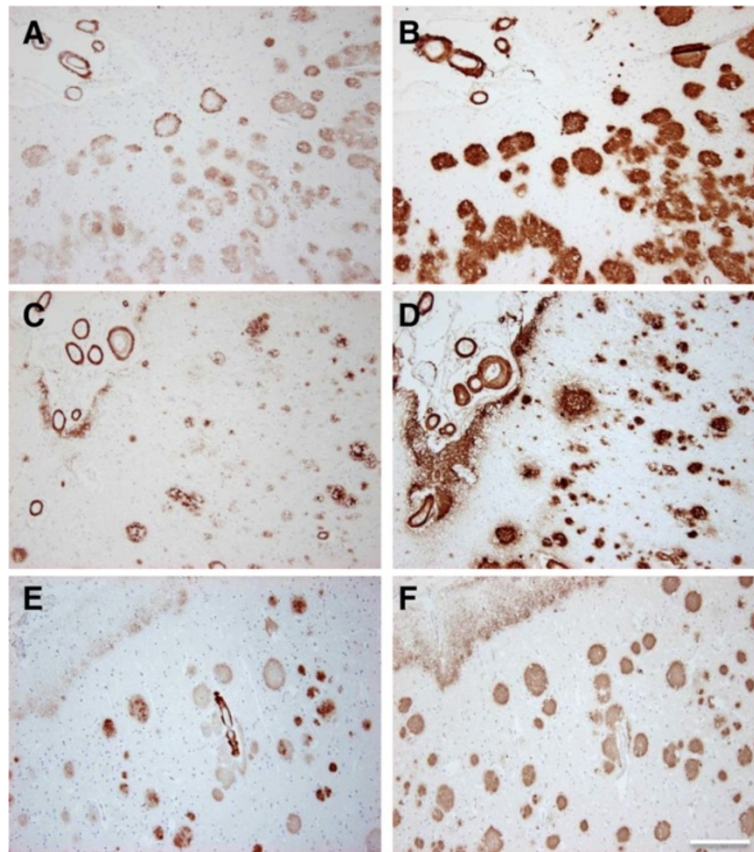


Figure 4 Immunohistochemical staining pattern of $A\beta_{5-x}$ in cortex of familial AD human brain. Vascular and parenchymal $A\beta_{5-x}$ -positive deposits were detected in parallel sections obtained from FAD cases including subjects with the Arctic (A) and Swedish mutations (C) in contrast to abundant 4G8-immunoreactivity in both cases (B, D). Sections of a PS1 Δ E9 mutation carrier probed with the AB5-3 antibody (E) showed equal or higher vascular $A\beta_{5-x}$ -immunoreactivity compared to A β 42 (F) whereas the $A\beta_{5-x}$ -parenchymal deposits were detected with lower intensity. Scale bar: 200 μ m.

In order to find out whether or not intracellular $A\beta_{5-x}$ is present early in the pathology, we used young APP/PS1KI (2-month-old) and homozygous 5XFAD (1.5-month-old) brain sections immunostained with the AB5-3 as both models harbor abundant intraneuronal $A\beta$ in contrast to young 3xTg mice, which accumulate mostly APP [24,25]. No intracellular $A\beta_{5-x}$ was evident, while the 24311 pan- $A\beta$ antibody consistently showed intraneuronal aggregations in both mouse models. (Figure 7). Therefore, $A\beta_{5-x}$ is not an early marker for neurodegeneration as it was only present in extracellular amyloid deposits.

Both diffuse and dense core plaques were positive. As it is known that neither 5XFAD, nor APP/PS1KI or 3xTg

develops CAA, we could not find any blood vessels stained with the $A\beta$ antibodies used.

Discussion

Nowadays it is evident that a great heterogeneity of $A\beta$ species exist that are deposited in AD brains including the full-length peptides, $A\beta_{1-40}$ and $A\beta_{1-42}$, and different C- and N- truncated isoforms (for example [26-28]). Especially N-truncated $A\beta$ peptides experienced a renaissance in the last decade with the development of new transgenic mouse models and novel $A\beta$ N-terminal specific antibodies for AD research [13,23]. N-truncated $A\beta_{x-42}$ species have attracted much attention triggering the accumulation of

Table 2 Transgenic AD-like mouse models in which $A\beta_{5-x}$ deposition has been analyzed

Mouse model	A β PP	PS1	Tau	Promoter	Age	$A\beta_{5-x}$ plaques	Ref.	Affected regions
APP/PS1KI	Swe, Lon	M233T, L235P	-	Thy1 (APP) PS1 knock-in	10 m	++	[20]	C, Hip, Th, PiC
5XFAD	Swe, Flo, Lon	M146L, L286V	-	Thy1 (APP, PS1)	12 m	++	[21]	C, Hip, Th, PiC
3xTg	Swe	M146V	P301L	Thy1 (APP, Tau) PS1 knock-in	18 m	+	[19]	Hip

Abbreviations: Swe Swedish, Flo Florida, Lon London, m age in months, C Cortex, Hip hippocampus, Th thalamus, PiC piriform cortex.

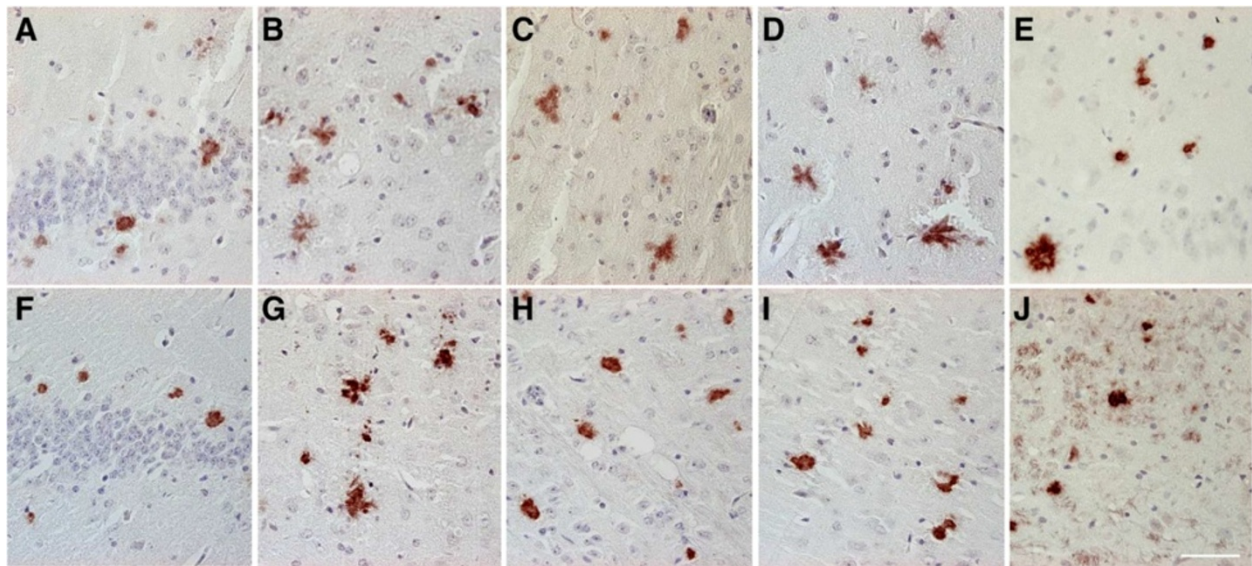


Figure 5 Immunohistochemical staining pattern of $A\beta_{5-x}$ in aged APP/PS1K1, 5XFAD and 3xTg transgenic mice. Parenchymal $A\beta_{5-x}$ extracellular deposits were detected with the AB5-3 polyclonal antibody in the APP/PS1K1 (A-D), 5XFAD (F-I) and 3xTg on hippocampus (E) and medulla (J). Larger regions were affected with plaques on hippocampus (A, F), cortex (B, G), thalamus (C, H) and piriform cortex (D, I) in the first two mouse models. Scale bar: 50 μ m.

$A\beta$ into neurotoxic aggregates. Ancolio *et al.* [29] was the first to show a selective and drastic increase of these species triggered by the mutation V715M APP770 in the APP gene, postulating that all $A\beta_{x-42}$ variants are main factors driving AD pathology.

The earliest evidence however for N-truncated peptides was observed by Masters *et al.* [30] demonstrating that $A\beta$ peptides starting with phenylalanine at position 4 are main components of amyloid plaques. $A\beta_{4-42}$ has been suggested to play an important role in the disease since it is deposited in early stages of the neuropathology

before other isoforms appear [31]. On the other hand, another prominent N-truncated variant, $A\beta_{pE3-42}$, was also suggested as a key player because of its limited degradation and remarkable stability [32]. It is becoming more and more accepted that the most prevalent $A\beta$ variants present in the brain regions affected by the disease are mainly the 42 ending variants $A\beta_{1-42}$, $A\beta_{pE3-42}$ and $A\beta_{4-42}$ (for example [26,28,33]). These $A\beta$ variants, particularly $A\beta_{4-42}$ and $A\beta_{pE3-42}$, are considered to be the most toxic due to their biochemical propensities to rapidly form stable oligomers [23].

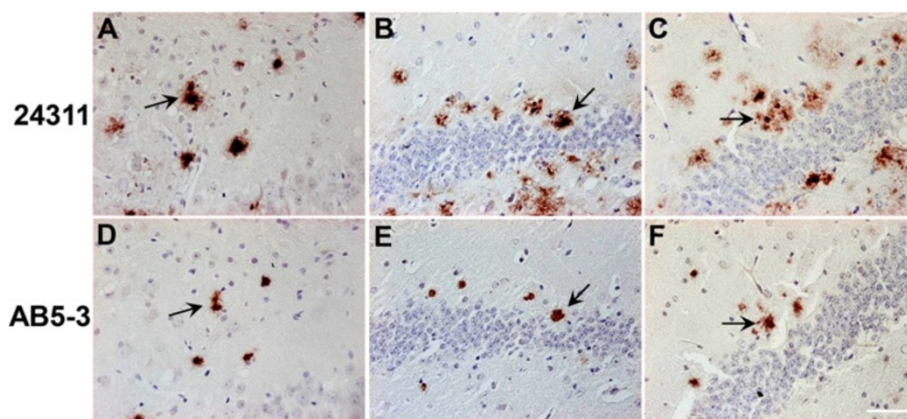


Figure 6 Analysis of consecutive sections of AD mouse models using the AB5-3 and 24311 antibodies. Extracellular plaques were detected in hippocampal brain sections of the 3xTg (A, D), 5XFAD (B, E) and the APP/PS1K1 (C, F) mouse models with the AB5-3 polyclonal antibody and the 24311 pan- $A\beta$ antibody. Parallel sections reveal $A\beta_{5-x}$ immunoreactivity localized in the core of the plaques (arrows point to the same amyloid plaque detected by the two different antibodies). Scale bar: 50 μ m.

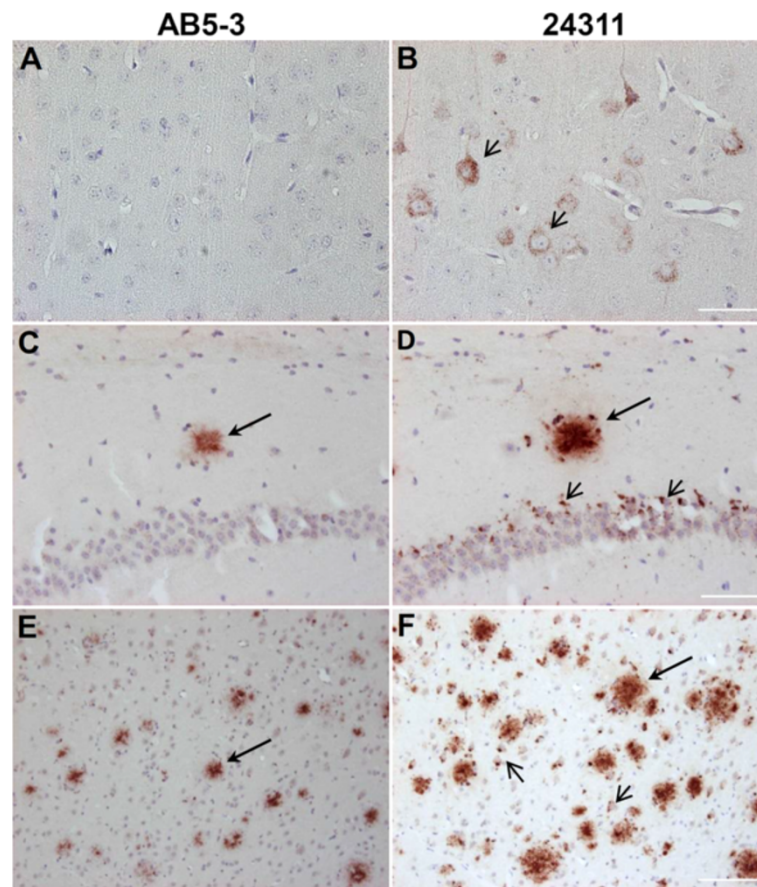


Figure 7 Young 5XFAD and APP/PS1K1 mice do not present intraneuronal $A\beta_{5-x}$ deposits. No intraneuronal $A\beta_{5-x}$ deposits were found in cortical brain sections of 1.5-month-old 5XFAD homozygous mice (A), as well as in hippocampal (C) and cortical sections of 2-month-old APP/PS1K1 mice (E). Parallel sections probed with the 24311 pan- $A\beta$ antibody demonstrated the presence of intraneuronal $A\beta$ immunoreactivity in both AD transgenic mouse models (small arrows: B, D, F). Abundant amyloid plaques were detected with both antibodies in young APP/PS1K1 mice (large arrows: C-F). Scale bar: A-D = 50 μ m; E, F = 100 μ m.

Despite the above mentioned importance of N-truncated peptides for AD, little is known about $A\beta_{5-x}$. It has been recognized to be present in $A\beta$ deposits of SAD patients [34]. Using mass spectrometry and Western blot of SAD cases and mass spectrometry of SAD and FAD cases (M146V PS1 or KM670/671NL APP), $A\beta_{5-40/42}$ was one of the detected N-truncated species [26-28,31]. Regarding transgenic mouse lines, mass spectrometry of immunoprecipitated $A\beta$ peptides have also provided evidence of the presence of $A\beta_{5-42}$. Casas *et al.* [20] reported $A\beta_{5-42}$ deposition in the APP/PS1KI mice. Our group detected $A\beta_{5-42}$ peptides in the 5XFAD mouse model [35].

The current report aims to provide a better understanding of a possible contribution of $A\beta_{5-x}$ in three different transgenic amyloid mouse models, SAD and FAD cases. Previous papers reported on a rabbit polyclonal [34] and a mouse monoclonal antibody [14] specific to the N-terminal end of $A\beta_{5-40/42}$, which bind to the epitope $A\beta_{5-12}$ (NH_2 -RHDSGYEVC-COOH). We have generated a rabbit polyclonal antibody, which specifically binds to $A\beta_{5-x}$ as shown

by dot blot and Western blot. The AB5-3 strongly detects $A\beta_{5-42}$ low molecular weight oligomers showing its specificity for peptides starting with an arginine at position 5, while other $A\beta$ variants starting at position 1, 3 (pyroglutamate) and 4 did not cross-react. While the polyclonal antibody AB5-3 reacted specifically with the free N-terminus of $A\beta_{5-42}$, we cannot exclude that it also may recognize $A\beta_{6-x}$ variants.

In agreement with the observations of the previously published polyclonal antibody [34], we detected parenchymal and vascular deposits in SAD. Vascular $A\beta_{5-x}$ seems to be present in most (80%) of the cases. Confirming published observations [34], the vascular $A\beta_{5-x}$ deposits often showed a stronger signal than aggregation in plaques of SAD cases. Immunostainings with the AB5-3 confirmed the presence of the $A\beta_{5-x}$ in extracellular deposits, however at a minor degree corroborating previous studies.

For the first time we present evidence that $A\beta_{5-x}$ immunoreactivity is abundant in extracellular plaques and vascular deposits in different FAD cases. The cases studied

included subjects expressing the APP Arctic and Swedish mutations, as well as the PS1 delta exon 9 mutation. Different patterns of A β_{5-x} aggregation were found among the different FAD cases. Previous data obtained from patients carrying the APP Arctic mutation reported ring-like plaques with A β peptides showing an accentuation of the contour and negative or weakly stained centers [36]. According to Nilsberth et al. [16] the Arctic mutation leads to an increase of A β protofibril formation, however the underlying molecular mechanism is still unknown.

Intraneuronal A β is a major risk factor in AD pathology triggering neuron loss [37-40]. During the last years, intraneuronal accumulation has been reported in several mouse models including APP_{SDL}PS1_{M146L} [41], APP_{SL}PS1_{M146L} [42], Tg2576 [43], 3xTg-AD [19], APP_{Arc} [44,45], 5XFAD [21], APP_{T714I} [46], APP_{SL}/PS1_{M146L} [47], APP/PS1KI [20,48,49], TBA2 mice expressing pyroglutamate modified A β_{3-42} [50] and in Tg4-42 expressing A β_{4-42} [23]. Furthermore, early intraneuronal A β accumulations have been detected in the homozygous 5XFAD mouse model at 1.5 months of age immediately preceding extracellular plaque deposition occurring at the age of 2 months [51]. Abundant intraneuronal A β has been demonstrated to correlate with a reduced number of neurons irrespectively of the extracellular A β aggregates in APP/PS1KI and in 5XFAD mice [40].

Using the AB5-3 no A β_{5-x} intraneuronal immunoreactivity was found in young mice APP/PS1KI and in 5XFAD mice. This observation indicates that A β_{5-x} may not contribute to trigger neuronal degeneration and appears late in AD pathology. Despite the fact that A β_{5-x} is a scarce variant in AD brains it was present in almost all sporadic cases and in virtually all the familial AD cases tested with a different degree regarding CAA and plaque deposition. Contrasting previous findings with abundant intraneuronal N-truncated A β_{4-x} in homozygous 5XFAD mice [51], A β_{5-x} appeared late in the amyloid cascade as there was no intraneuronal staining in young APP/PS1KI and homozygous 5XFAD mice. A variety of different N-truncated A β peptides besides A β_{5-x} (Arg-5) have been identified in AD brains including Ala-2, pyroglutamylated Glu-3, Phe-4, His-6, Asp-7, Ser-8, Gly-9, Tyr-10 and pyroglutamylated Glu-11 [26-31,52-55]. We cannot rule out however that the polyclonal antibody AB5-3 may cross-react with other N-truncated A β variants not studied here that may be more prevalent than Arg-5 in amyloid plaques.

Conclusions

Although a considerable amount of A β_{5-x} can be found in transgenic AD mouse models, SAD and FAD cases, no intracellular A β_{5-x} deposits of these peptides were found neither in the different mouse lines nor in the human cases. Due to the lack of intracellular A β_{5-x} , it is unlikely to participate in early events of the AD pathology. This

indicates that A β_{5-x} might participate in extracellular precipitation of A β peptides within plaques and is therefore not likely to be involved in triggering neuronal loss.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EAG performed experiments and wrote the paper, YB, BCR and OW contributed to experiments, LL, MI, AP and AV-A contributed with neuropathological and clinical expertise, TAB designed the study and wrote the paper. All authors read and approved the final manuscript.

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