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Amino-terminally elongated Aβ peptides are generated by the secreted metalloprotease ADAMTS4 and deposit in a subset of Alzheimer's disease brains

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Abstract

Aims: The aggregation and deposition of amyloid-β (Aβ) peptides in the brain is thought to be the initial driver in the pathogenesis of Alzheimer's disease (AD). Aside from full-length Aβ peptides starting with an aspartate residue in position 1, both N-terminally truncated and elongated Aβ peptides are produced by various proteases from the amyloid precursor protein (APP) and have been detected in brain tissues and body fluids. Recently, we demonstrated that the particularly abundant N-terminally truncated Aβ4–x peptides are generated by ADAMTS4, a secreted metalloprotease that is exclusively expressed in the oligodendrocyte cell population. In this study, we investigated whether ADAMTS4 might also be involved in the generation of N-terminally elongated Aβ peptides.

Methods: We used cell-free and cell-based assays in combination with matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF) and electrochemiluminescence sandwich immunoassays to identify and quantify N-terminally elongated Aβ peptide variants. Antibodies against these Aβ variants were characterised by peptide microarrays and employed for the immunohistochemical analyses of human brain samples.

Results: In this study, we discovered additional ADAMTS4 cleavage sites in APP. These were located N-terminal to Asp-(1) in the Aβ peptide sequence between residues Glu-(-7) and Ile-(-6) as well as Glu-(-4) and Val-(-3), resulting in the release of N-terminally elongated Aβ-6-x and Aβ-3-x peptides, of which the latter serve as a component in a promising Aβ-based plasma biomarker. Aβ-6/-3-40 peptides were detected in supernatants of various cell lines and in the cerebrospinal fluid (CSF), and ADAMTS4 enzyme activity promoted the release of Aβ-6/-3-x peptides. Furthermore, by immunohistochemistry, a subset of AD cases displayed evidence of extracellular and vascular localization of N-terminally elongated Aβ-6/-3-x peptides.

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Discussion: The current findings implicate ADAMTS4 in both the pathological process of Aβ peptide aggregation and in the early detection of amyloid pathology in AD.

KEYWORDS
Aβ, ADAMTS4, Alzheimer's disease, amyloid precursor protein, immunoassay, immunohistochemistry, immunoprecipitation, mass spectrometry

INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disorder, accounting for ~70% of all dementia cases in individuals older than 60 years of age [1]. Neuropathologically, the disease is characterised by the extracellular deposition and accumulation of proteinaceous aggregates within the human brain, referred to as amyloid plaques. Intracellular assemblies of the hyperphosphorylated protein Tau, the neurofibrillary tangles, represent the second major neuropathological hallmark [2]. The amyloid plaques are mainly composed of aggregated amyloid-β (Aβ) peptides, which are derived from the large amyloid precursor protein (APP) in a normal physiological process via successive proteolytic cleavage events. The "canonical" Aβ peptide sequence has a length of 40–42 amino acids, but numerous Aβ peptide variants differing in the exact length of their amino and carboxy termini have been detected. Under physiological conditions and in many cellular systems, almost 90% of the total Aβ produced is Aβx–40, with Aβx–42 accounting for less than 10% [3]. Most of these peptides represent "full-length" Aβ species (Aβ1–40/Aβ1–42), starting with an aspartic acid (Asp-(1)) as the amino-terminal residue, and they originate from β-secretase (BACE1) cleavage at the N-terminus [4, 5]. However, mass spectrometry studies of human brain tissue samples have revealed the presence of a variety of C- and N-terminally truncated Aβ peptides (e.g., Aβ1–38 and Aβ4–42) or post-translationally modified Aβ variants (such as AβpE3–42) [6–8]. N-terminally truncated Aβ peptides starting with a phenylalanine residue at position 4 are particularly abundant in the human AD brain [6, 8–11]. Masters and colleagues found that >60% of the formic acid extractable Aβ peptides from amyloid plaque cores that were accessible to Edman protein sequencing started with phenylalanine (Phe(4)) [12]. Recently, we identified an enzymatic mechanism for the generation of Aβ4–x peptides and demonstrated that the secreted metalloprotease ADAMTS4 (a disintegrin and metalloproteinase with thrombospondin motifs 4) facilitates APP cleavage between Glu(3) and Phe(4) in vitro and in vivo [13]. In the human brain, this enzyme is exclusively expressed in oligodendrocytes [13, 14], and ADAMTS4 has been shown to be essential for Aβ4–40 secretion in oligodendrocyte precursor cells. Oligodendrocytes are of particular interest as myelin dysfunction has been identified as a potent driver of amyloid deposition in AD mouse models [15]. Of note, large-scale genome-wide association studies identified ADAMTS4 as a potential novel AD risk locus [16, 17].

Aside from full-length and N-terminally truncated Aβ peptides, N-terminally elongated species such as Aβ-3-40 (corresponding to APP669-711) have been identified [18, 19]. While these N-terminally elongated Aβ peptides have been studied far less, the Aβ-3-40/Aβ1–42 ratio in blood plasma has been reported as an accurate and easily accessible peripheral biomarker of cerebral Aβ accumulation [20, 21]. In addition, we detected Aβ-3-40 peptides in the human cerebrospinal fluid (CSF) [19], but the molecular origin of these peptides remained unknown. When revisiting previously published mass spectra of Aβ variants from cells with ectopic expression of ADAMTS4 [13], we observed signals consistent with the presence of Aβ-3-40 peptides, indicating that ADAMTS4 might also be involved in the generation of these N-terminally elongated Aβ species [22]. In the current study, we employed a previously developed electrochemiluminescence sandwich immunoassay [23] and immuno-precipitation (IP) followed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS), to identify N-terminally elongated Aβ-6-x and Aβ-3-x peptides and to determine their levels in the supernatants of a variety of cell lines as well as human CSF. Furthermore, in both cell-free and cell-based assays, we demonstrated that ADAMTS4 enzyme activity resulted in the elevated release of N-terminally elongated Aβ peptide species. Finally, we performed a detailed immunohistochemical analysis of human brain samples and provided the first evidence that a subset of AD cases displays extracellular and vascular localization of N-terminally elongated Aβ peptide variants.

Key points

- The metalloproteinase ADAMTS4 generates N-terminally elongated Aβ species.
- N-terminally elongated Aβ variants have been detected in cerebrospinal fluid.
- Immunohistochemical analyses indicated deposition of N-terminally elongated Aβ variants in the brains of AD patients.

MATERIAL AND METHODS

Human brain and CSF samples

Paraffin-embedded human brain samples from sporadic AD ($n = 30$; mean age 78.7 ± 12.11, average post-mortem interval [PMI] = 339.7 min ± 88.1 min; 18 females, 12 males), nondemented controls (NDC; $n = 7$; mean age 87.57 ± 4.31, average PMI = 310.3 min ± 60.6 min; six females, one male) and Down

syndrome (DS; $n = 3$; mean age 61.0 ± 3.00, average PMI = 315 min ± 57.6 min; two female, one male) (Table S1) were obtained from the Netherlands Brain Bank (NBB), and all material and data collected by the NBB are obtained on the basis of written informed consent. Samples from the medial frontal and superior temporalis gyrus regions of the brain were used in the analysis. All procedures involving human tissue have been approved by the ethical committee at the University Medical Center Göttingen (Protocol number 12/1/15). The CSF sample pool was prepared by combining several CSF samples from the local biobank of the Department of Psychiatry at the University Medical Center Goettingen. The collection of biological samples and clinical data in a pseudonymised fashion and their use in biomarker studies was approved by the ethics committee of the University Medical Center Goettingen (Protocol number 9/2/16).

Generation of cell lines with doxycycline-inducible ADAMTS4 expression

HEK293 human embryonic kidney cell lines with inducible ADAMTS4 expression were generated using a lentiviral, tetracycline-controlled expression system and have been described previously [13]. The expression system was a gift by Dr Eric Campeau and consisted of the lentiviral expression vectors pLenti CMVtight Hygro DEST (Addgene plasmid #26433) and pLenti CMV rtTA3 Blast (Addgene Plasmid #26429), which encoded the reverse tetracycline-controlled transactivator 3 (rtTA3). Full-length human ADAMTS4 with a C-terminal V5-tag was cloned into pLenti CMVtight Hygro DEST by Gateway cloning (ThermoFisher Scientific). Viral particles were produced in 293FT cells using a third-generation lentivirus packaging system [24]. To generate double-stable cell lines expressing both rtTA3 and tetracycline-inducible ADAMTS4, either HEK293 cells with endogenous APP expression, or HEK293 cells with stable overexpression of the human APP695 isoform (HEK-APP695wt cells), or HEK293 cells with stable overexpression of the human APP695 isoform containing the "Swedish" double-mutation (HEK-APP695sw cells, both APP-overexpressing HEK293 cell lines were a gift of Dr Edward Koo, UC San Diego, USA) were first infected with lentiviral particles expressing rtTA3, and stable mass cultures were selected with blasticidin. Subsequently, rtTA3-expressing cells were infected with ADAMTS4 lentiviral particles, and stable single-cell clones were isolated with hygromycin selection and limiting dilution cloning. Double-stable cell lines were maintained in antibiotic selection medium containing DMEM with 10% FCS (v/v), 1% sodium pyruvate (v/v), 100 U/mL penicillin/streptomycin, blasticidin and hygromycin (all media components from ThermoFisher Scientific). Additional cell lines with inducible expression of ADAMTS4 based on neuroglioma cells with stable overexpression of APP695wt (H4-APP695wt cells, a gift by Dr Karlheinz Baumann, Roche Innovation Center Basel, Switzerland) and Chinese hamster ovary cells with stable overexpression of APP751wt (CHO-APP751wt, clone 7WD10, a gift by Dr Edward Koo) were generated accordingly.

Aβ enzyme-linked immunosorbent assays

To measure the concentrations of N-terminally truncated Aβ4–40 peptides and of Aβx–40 and Aβx–42 variants starting with an aspartic acid residue (Asp-(1)) in conditioned cell culture supernatants, previously described sandwich enzyme-linked immunosorbent assays (ELISAs) were used [11, 13, 25]. Standard curves were generated with synthetic Aβ peptides (JPT). To detect Aβx–40 and Aβx–42 variants starting with Asp-(1), Ala-(2) or Glu-(3) at the N-terminus, the monoclonal antibody (mAb) IC16 [26] was used for capture and combined with antibodies mAb BAP-24 [27] and mAb BAP-15 [28] against the C-termini of Aβx–40 and Aβx–42 (the detection antibodies were a gift of Dr Karlheinz Baumann). As shown previously with a capillary isoelectric focusing immunoassay, mAb IC16 detected synthetic Aβx–40 variants starting with Asp(1), Ala(2) and Glu(3) but did not detect Aβ4–40 under the tested conditions [11]. However, earlier IP-MS data showed no evidence for the presence of Aβ2–x or Aβ3–x in conditioned media from the cell lines under investigation [13]. Therefore, for the sake of readability, we refer to the Aβ peptides measured with the IC16 ELISAs as Aβ1–40 and Aβ1–42. To detect N-truncated Aβ4–40 peptides, the Aβx–40 C-terminus specific mAb BAP-24 was used as a capture antibody and combined with the Aβ4–x specific mAb 18H6 [29] as a detection antibody; 96-well high-binding microtiter plates (Greiner Bio-One) were incubated overnight at 4°C with the capture antibodies in phosphate-buffered saline (PBS), pH 7.2. After excess capture antibody was removed, tissue culture media or Aβ peptide standards (in PBS, 0.05% Tween-20 (v/v), 1% BSA (w/v)) were added. Then, the detection antibodies labelled with horseradish peroxidase using the Pierce EZ-Link Plus Activated Peroxidase kit (ThermoFisher Scientific) and diluted in PBS, 0.05% Tween-20 (v/v), 1% BSA (w/v) were added to each well and incubated overnight at 4°C. Plates were washed three times with PBS containing 0.05% Tween-20 (v/v) and once with PBS. Subsequently, 50 µL of trimethylbenzidine ELISA peroxidase substrate (ThermoFisher Scientific) was added and incubated for 1–5 min at RT in the dark. The reaction was terminated by adding 50 µL of 2-M H_2SO_4, and the absorbance was recorded using a Paradigm microplate reader (Beckman Coulter) at 450 nm.

In vitro ADAMTS4 proteolytic cleavage assay

To analyse ADAMTS4 cleavage products, 440 ng of the synthetic Aβ model peptide APP649–687/Aβ-23-16 (H_2N-GLTTRPGSGLTNIK-TEEISEVKMDAEFRHDSGYEVHHQK-CONH$_2$; Asp(1) of the Aβ sequence is underlined) [23] were incubated with 200-ng recombinant ADAMTS4 (#4307-AD-020, R&D Systems) in 20-mM HEPES, 200-µM $CaCl_2$, pH 7. Inhibition of ADAMTS4 was achieved by the addition of the broad-spectrum metalloprotease inhibitor Batimastat (BB94, #S7155, Selleckchem) at a final concentration of 10 µM. A sample without substrate peptide was used to control for the occurrence of proteolytic peptides resulting from autolytic cleavage of ADAMTS4. An aliquot of each sample was taken immediately after

mixing ($t = 0$ h) and digestion was stopped by the addition of trifluoroacetic acid (TFA) at 5% final concentration. In the remaining samples, digestion was allowed to proceed for 4 h at 37°C and then stopped ($t = 4$ h). For the analysis of the proteolytic cleavage products by MALDI-TOF-MS, samples were prepared on α-cyano-4-hydroxycinnamic acid (CHCA, #70990, Sigma Aldrich) matrix according to the thin-layer affinity method and analysed on a Bruker ultrafleXtreme mass spectrometer using standard procedures [30, 31].

Electrochemiluminescence immunoassay to measure N-terminally elongated Aβ species in cell culture supernatants

The concentrations of N-terminally elongated Aβ variants in cell culture supernatants were measured with a chemiluminescence sandwich immunoassay as described previously [23]. In brief, the assay was developed and carried out on the MSD technology platform employing MSD Gold 96-well small spot plates precoated with streptavidin (Meso Scale Discovery, MSD, Rockville, MD, USA). Biotinylated mAb 101-1-1 was used as a capture antibody and Sulfo-Tag-Aβ40 (MSD) served as a detection antibody. Standard curves were prepared using synthetic Aβ-3-40 peptides and data analysis including standard curve calculations were carried out with the Discovery Workbench 4.0.12 software package (MSD).

Peptide microarray: synthesis, quality control and immunoassay

The APP sequence 649–722 (UniProtKB: P05067; Aβ numbering -23-51) was displayed in microarray format as a 15-mer overlapping peptide library. Peptide arrays were synthesised [32–34] using a MultiPep RSi robot (CEM GmbH, Germany) on cellulose discs containing 9-fluorenylmethyloxycarbonyl-β-alanine (Fmoc-β-Ala) linkers. Synthesis was carried out by deprotecting the Fmoc-group using 20% piperidine in dimethylformamide (DMF). Peptide chains were elongated using a coupling solution consisting of amino acid building blocks (IRIS Biotech GmbH, Germany) with Oxyma and diisopropylmethanediimine in DMF (1:1:1). Coupling steps were carried out 3 times (30 min), followed by capping (4% acetic anhydride in DMF). Discs were transferred into 96 deep-well plates for the work-up. Side chains were deprotected using 90% trifluoracetic acid (TFA), 2% dichloromethane, 5% H_2O and 3% triisopropylsilane (150 µL/well) for 1 h at room temperature. Afterwards, the deprotection solution was removed, and the discs were solubilised overnight at RT, while shaking, using a solvation mixture containing 88.5% TFA, 4% trifluoromethanesulfonic acid, 5% H_2O and 2.5% triisopropylsilane (250 µL/well). The resulting peptide-cellulose conjugates (PCCs) were precipitated with ice-cold ether (700 µL/well) and spun down at 2000 × *g* for 10 min at 4°C, followed by two additional washes of the formed pellet with ice-cold ether. The resulting pellets were dissolved in DMSO (250 µL/well).

LC-MS was carried out using peptide quality controls that were cleaved from the solid support. To ensure cleavage, a Rink amide linker (Iris Biotech GmbH, Germany) was introduced during the second coupling cycle after the Fmoc-β-Ala linker. In an acidic environment, the quality controls were cleaved off the solid support. To isolate the quality controls, 150 µL of the supernatant was transferred to 1.5-mL reaction tubes, followed by the addition of 700 µL of diethyl ether. The samples were then vortexed, and the peptides were allowed to precipitate by incubation at −20°C overnight. After centrifugation at 13,300 × *g* and 4°C for 10 min, the supernatant was discarded, and 500 µL of diethyl ether was added. The mixture was vortexed, centrifuged for 10 min, and the supernatant was decanted. This process was repeated twice, and the peptides were left to dry for 60 min. Finally, the Rink amides were dissolved in 50 µL of 50% acetonitrile; 0.1% formic acid (v/v) and vortexed briefly before centrifugation at 13,300 × *g* and RT. For analysis, the quality controls were diluted 1:3 and sent to LC-MS (Agilent Technologies, USA).

PCCs solutions were mixed 2:1 with saline–sodium citrate buffer (150-mM NaCl, 15-mM trisodium citrate, pH 7.0) and transferred to a 384-well plate. For the transfer of the PCC solutions to white-coated CelluSpot blank slides (76 × 26 mm, Intavis AG Peptide Services GmbH and CO. KG), a SlideSpotter (CEM GmbH) was used. After completion of the printing procedure, slides were left to dry for a minimum of 3 h. The microarray slides were blocked for 60 min in 5% (w/v) skimmed milk powder (Carl Roth) 0.05% Tween-20 in PBS. After blocking, the slides were incubated for 30 min with a solution of blocking buffer and mAbs 101-1-1 or 14-2-4 (at a final concentration of 1 µg/mL). They were then washed 3× with PBS 0.05% Tween20 for 1 min. Antibodies were detected using goat anti-mouse IgG-HRP (Thermo Fisher Cat. No 31470). The chemiluminescence readout was detected with an Azure imaging system c400 (lowest sensitivity) using SuperSignal West Femto maximum sensitive substrate (Thermo Scientific GmbH, Germany).

Immunoprecipitation and mass spectrometry

The monoclonal antibodies mAb 6E10 (BioLegend, Cat. No SIG-39320) (RRID:AB_662798) or mAb 101-1-1 [23] were covalently coupled to Dynabeads M-270 Epoxy (Invitrogen/ThermoFisher Scientific Cat. No. 14311 D), essentially following the manufacturer's instructions provided with the coupling kit. For specific use in IP-MS, Tween-20 was omitted from all wash and storage buffers.

In brief, 5 mg of M-270 epoxy beads were weighed into a 1.5-mL reaction vial and washed with 1 mL of buffer C1 (kit component). The beads were immobilised on a magnet, and the supernatant was discarded. Buffer C1 and 50 µg of IgG (total volume of buffer C plus antibody stock solution = 250 µL) were added to the beads. After gentle mixing by pipetting, 250 µL of buffer C2 (kit component) was added and mixed. Then, the mixture was incubated overnight at 37°C on a rotating mixer. On the next day, the beads were immobilised on a magnet, the supernatant was removed, and the beads were washed 1× with 800 µL of buffer HB (kit component), 1× with 800 µL of

buffer LB (kit component) and 2× with 800 μL of PBS. After a final 15-min wash with PBS, the beads were immobilised on a magnet, and the supernatant was discarded. For storage at 4°C, the beads were resuspended in 500 μL of PBS plus 5 μL of 2% (w/v) NaN_3 (final concentration: 0.02% NaN_3).

For immunoprecipitation of Aβ peptides (essentially according to [19, 20]), 250 μL of cell culture supernatant was mixed with an equal volume of 100-mM Tris-HCL, pH 7.4 containing 0.2% (w/v) nonyl-β-d-thiomaltoside (NTM) and 0.2% (w/v) n-dodecyl-β-d-maltoside (DDM) and 15 μL of functionalized Dynabeads M270 epoxy carrying mAb 6E10 or mAb 101-1-1. After overnight incubation at 4°C with constant mixing, the beads were washed 5× with 0.5 mL of 50-mM Tris-HCl, pH 7.4, 150-mM NaCl, 0.1% (w/v) DDM, 0.1% (w/v) NTM, resuspended in 0.5 mL of 50-mM ammonium acetate (pH approximately 7.0) and immediately forwarded to sample preparation for mass spectrometry. For that purpose, the beads were immobilised on a magnet, the supernatant was removed, and the beads were washed 1× with 0.5 mL of 50-mM ammonium acetate, and 1× with 0.5-mL H_2O.

Bound Aβ peptides were eluted in 2.5 μL of 70% acetonitrile containing 5-mM HCl. When quantification was desired, the eluent additionally contained a 50-nM final concentration of the synthetic Aβ5–38(D23S) peptide featuring an Asp to Ser amino acid substitution at position 23 [35] to be used as a standard for spectra normalisation.

In order to identify N-terminal elongated Aβ variants present in human CSF, pooled samples were preconcentrated and subjected to IP-MS. Aliquots of CSF samples were thawed at room temperature and mixed vigorously for 5× 10 s. Insoluble material was removed by centrifugation for 10 min at 10,000 × *g* using a fixed angle rotor. After merging each of the supernatants, a final volume of 10 mL of pooled CSF was obtained. Preconcentration of the sample was performed using an Amicon Ultra-4 Centrifugal Filter with 3000 MWCO (Millipore Cat. No. UFC800308). In brief, 4 mL of pooled CSF was added to the Amicon Ultra filter device and centrifuged in a swing-bucket rotor at 4000 × *g* for 20 min. The flowthrough was discarded and another 4 mL of pooled CSF were added, and the process was repeated. The final 2 mL of sample were added and centrifuged for 15 min. To recover the concentrated solute, the sample was immediately removed from the bottom of the filter device (approximately 500 μL were obtained). CSF fluid from the flowthrough was added to obtain a final volume of 1 mL. The final concentrated CSF sample was immediately mixed with 1 mL of 100-mM Tris-HCL, pH 7.4 containing 0.2% (w/v) NTM and 0.2% (w/v) DDM and 15 μL of functionalized 101-1-1 Dynabeads M270 epoxy and incubated overnight at 4°C with constant mixing. The following day, the beads were washed 5× with 0.5 mL of 50-mM Tris-HCl, pH 7.4, 150-mM NaCl, 0.1% (w/v) DDM, 0.1% (w/v) NTM, resuspended in 0.5 mL of 50-mM ammonium acetate (pH approximately 7.0) and immediately forwarded to sample preparation for mass spectrometry.

Analysis by MALDI-TOF-MS was essentially performed as described recently [19]. Briefly, the eluates (0.5 μL) were spotted onto a prestructured MALDI sample support (MTP AnchorChip 384 BC; Cat. No. 8280790, Bruker, Bremen, Germany), followed by the addition of 0.5-μL matrix solution consisting of 5 mg/mL CHCA in 50% acetonitrile/0.05% TFA. A 1:1 mixture of Peptide Calibration Standard II (Cat. No. 8222570, Bruker, Bremen, Germany) and PepMix2 (Cat. No. C102, LaserBio Labs, Valbonne, France) was used as calibrant. The samples were dried in a vacuum desiccator and a total of 5000 mass spectra per spot were recorded using an ultrafleXtreme MALDI-TOF/TOF mass spectrometer operated under the software flexControl 3.4 (Bruker, Bremen, Germany). Depending on the desired characteristics of the mass spectrometric data, the instrument was either operated in reflector mode for the annotation of Aβ species based on high resolution and mass accuracy, in linear mode for quantification purposes or in a dedicated linear mode with laser energy (30%–40%) and detector voltage (20%–30%) boost for maximal sensitivity.

Immunohistochemistry of paraffin-embedded brain samples

Immunohistochemistry was performed on 4-μm paraffin sections as previously described [36]. In brief, after a deparaffinisation step in Roticlear (Carl Roth) and rehydration in a descending series of ethanol (100%, 95%, 70%), blocking of endogenous peroxidases was carried out by incubation in 0.3% H_2O_2 in PBS for 30 min. For antigen retrieval, sections were boiled in 0.01-M citrate buffer (pH 6.0) and incubated in 88% formic acid. Nonspecific binding sites were blocked by treatment with PBS containing 4% skim milk and 10% foetal calf serum for 1 h at room temperature, prior to the incubation with the primary antibodies. The following antibodies were used for the detection of different Aβ variants: 24311 (rabbit pAb [37], 1:500) against an epitope located in the N-terminal region of Aβ; 18H6 (mouse mAb, 1.9 μg/mL [29]) for the detection of Aβ starting with Phe at position 4; mouse mAbs 101-1-1 and 14-2-4 raised against Aβ-3-5 and displaying high preference for N-terminally elongated Aβ peptides (both 1 μg/mL) [23]. In addition, 22C11, a mouse mAb detecting the N-terminus of human APP (Millipore, #MAB348, 2 μg/mL; RRID: AB_94882) and IG7/5A3 (5 μg/mL) raised against an epitope in the central part of APP [38] were used. Biotinylated secondary anti-mouse and anti-rabbit antibodies (Jackson Immuno Research) were used in a 1:200 dilution. Staining was visualised using the ABC method with a Vectastain kit (Vector Laboratories) and diaminobenzidine (DAB) as a chromogen. Counterstaining was carried out with haematoxylin. In order to perform a semiquantitative analysis of the staining, the sections were evaluated using a BX51 microscope (Olympus) equipped with a Moticam Pro 282 camera (Motic) in a blinded fashion. The following semiquantitative scoring criteria were used to assess parenchymal and vascular staining: 0 = no staining, 0.5 = barely detectable staining, 1 = weak staining, 2 = moderate staining and 3 = extensive staining. Heat maps were plotted using GraphPad Prism 9.5.1.

Antibody preadsorption with synthetic Aβ peptides

Antibody preadsorption with synthetic Aβ-3-40 and Aβ1–40 peptides was carried out as described previously [39]. In brief, 1 μg of 101-1-1 was incubated with either Aβ-3-40 or Aβ1–40 in a fivefold excess (5 μg) in a total volume of 1 mL PBS including 10% FCS at room temperature for 3 h with constant rotation. As a control condition, 1 μg of 101-1-1 was incubated in 1 mL 0.01-M PBS without synthetic peptides. At the end of the incubation period, vials were centrifuged at 13.000 rpm for 5 min and paraffin-embedded parallel sections (4 μm thickness) from human AD cases were incubated overnight at 4°C with the supernatants. The next day, sections were washed in PBS including 1% Tergitol and were incubated with biotin-conjugated anti-mouse antibodies (Jackson Immunoresearch, 1:200) in PBS including 10% FCS. Staining was visualised with a Vectastain kit (Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine (DAB) as chromogen and counterstained with haematoxylin.

Statistical analysis

Differences between groups were tested with unpaired *t* tests, multiple *t* tests or one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc tests. All data are displayed as means ± standard deviation (SD). Significance levels are given as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. All calculations were performed using GraphPad Prism version 9.5.1 for Windows (Graph Pad Software, San Diego, USA).

RESULTS

ADAMTS4 cleaves between residues Glu-(-4) and Val-(-3) N-terminal of Asp-(1) in the Aβ sequence

As a starting point for this study, we revisited our previously published Aβ IP-MS data obtained from conditioned media of HEK293 cells with stable overexpression of human, wild type APP695 and doxycycline (DOX) inducible expression of human ADAMTS4 [13]. In this context, we recognised a so far unassigned signal that was increased upon DOX induction of ADAMTS4 expression (Supplementary Figure S3D in [13]; see mass range above the Aβ1–42 signal) and was absent in IP-MS spectra from cells with stable co-expression of Swedish mutant APP695sw and ADAMTS4 (Figure 1D in [13]). As its observed mass corresponded to that of N-terminally elongated Aβ-3-40 (aka APP669–711, Figure S1), which we recently identified in human cerebrospinal fluid [19], we reasoned that ADAMTS4 might be involved in the proteolytic generation of N-terminally elongated Aβ peptides.

To further investigate whether this is the case, we initially performed a series of in vitro experiments to confirm that ADAMTS4 is indeed able to cleave APP between residues Glu-(-4) and Val-(-3) (Aβ sequence numbering). To this end, we made use of a synthetic model peptide comprising the first 16 amino acid residues of the canonical Aβ sequence with an extension of 23 amino acids at the N-terminus (Aβ-23-16 corresponding to APP649–687; see Methods for the sequence) [23]. This substrate peptide was incubated with recombinant ADAMTS4 in the presence or absence of the broad-spectrum metalloprotease inhibitor Batimastat (BB94) for up to 4 h, followed by MALDI-TOF-MS analysis. As expected, no major changes in the signals for Aβ-23-16 were observed when the substrate peptide was incubated in the absence of ADAMTS4 (negative control) (Figure 1A) or in the presence of ADAMTS4 and the BB94 inhibitor (Figure 1C). Confirming our previous findings that ADAMTS4 generates N-terminally truncated Aβ4–x peptides [13], MS analysis of ADAMTS4-digested Aβ-23–16 showed the presence of the peptide fragments Aβ4–16 and Aβ-23-3 after 4 h (Figure 1B). Importantly, this was accompanied by the detection of Aβ-23-(-4) and Aβ-3-16 peptide variants, supporting the proposition that ADAMTS4 cleaves APP between residues Glu-(-4) and Val-(-3) to generate N-terminally elongated Aβ-3-x peptides (Figure 1B). Unexpectedly, an increase of Aβ5–16 was also observed upon incubation of the Aβ-23–16 substrate peptide with recombinant ADAMTS4 (Figure 1B).

Aβ-3-x peptides are detectable in cell culture supernatants and can be generated by ADAMTS4

To corroborate these findings in a cell-based model, we employed previously generated HEK293 cell lines with stable overexpression of either APP695wt or APP695sw and inducible expression of ADAMTS4 [13]. Western blot analysis confirmed the successful induction of ADAMTS4 expression upon doxycycline treatment (Figure S3). First, to validate this ADAMTS4 expressing experimental model, we examined whether ADAMTS4 overexpression affected the secretion of N-terminally truncated Aβ4–x peptides and of Aβ peptides with an intact N-terminus (Aβ1–x). In agreement with our earlier study [13], measurements of Aβ1–40 and Aβ1–42 did not show significant differences in HEK293-APP695wt/ADAMTS4 cells after induction of ADAMTS4 expression (Figure 2A). In contrast, a significant increase in Aβ4–40 levels was detected ($p < 0.001$; Figure 2A), consistent with the reported ADAMTS4 cleavage site between Glu(3) and Phe(4) [13]. Similar results were obtained with HEK293-APP695sw/ADAMTS4 cells (Figure 2B). However, Aβ1–40 levels were substantially higher in conditioned media of APP695sw expressing cells, and induction of ADAMTS4 expression resulted in significantly reduced Aβ1–40 levels ($p < 0.01$), potentially due to competition between BACE1 and ADAMTS4 as the Swedish mutation facilitates the processing of APP in the amyloidogenic pathway (Figure 2B).

To unequivocally confirm the presence of N-terminally elongated Aβ-3-40 peptides in conditioned supernatants of HEK293-APP695wt/ADAMTS4 cells, media were subjected to a single round of magnetic bead immunoprecipitation and subsequent analysis by MALDI-TOF-MS. Aβ peptides were immunoprecipitated with anti-mouse magnetic beads covalently functionalized with mAb 6E10, an antibody shown to detect a variety of Aβ variants with different

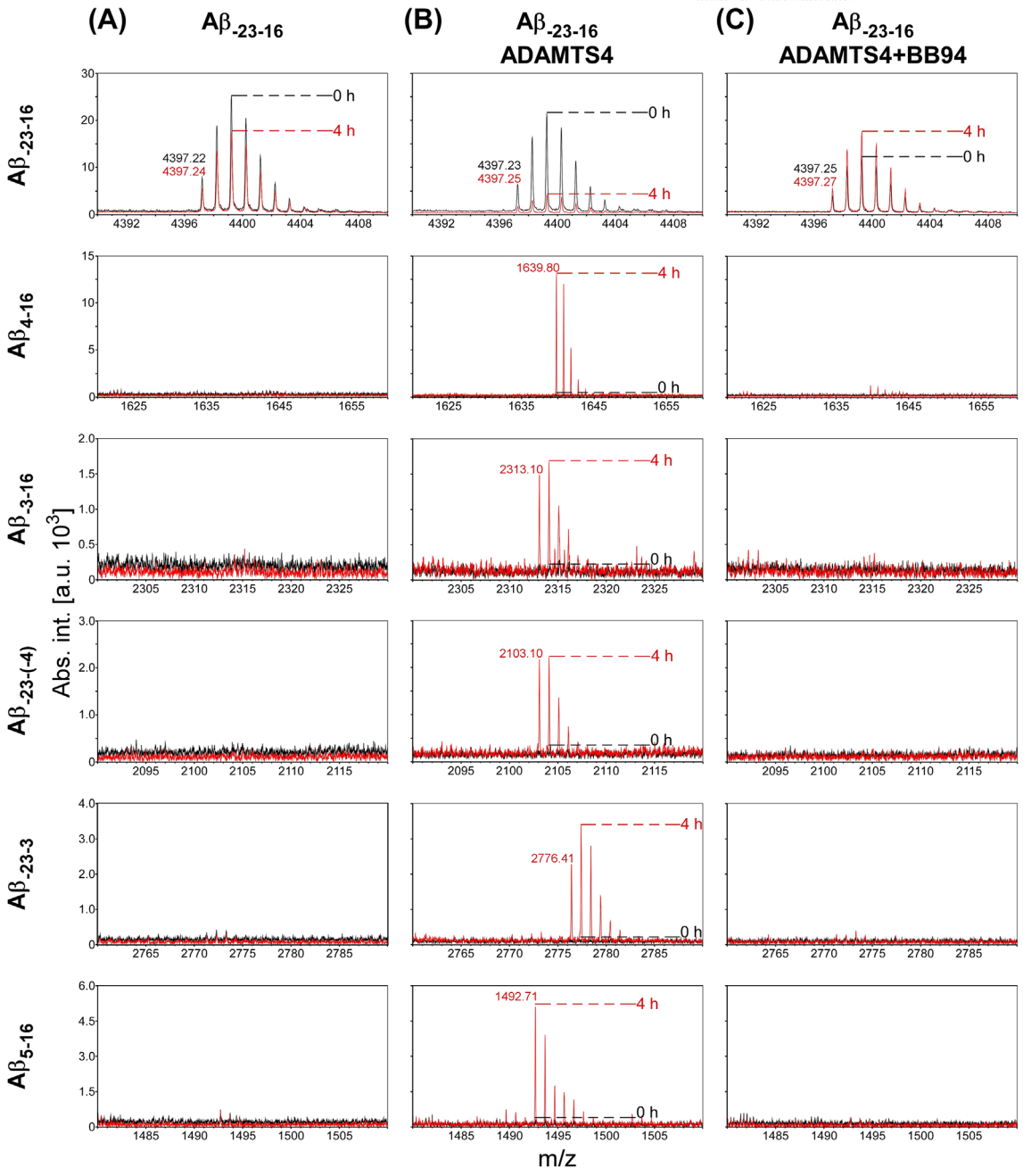

FIGURE 1 MALDI-TOF-MS analysis of the cleavage products after in vitro hydrolysis of the synthetic substrate peptide Aβ-23-16 by recombinant ADAMTS4. Aβ-23-16 was incubated without ADAMTS4 (mass spectra in A), with recombinant ADAMTS4 (B) or with ADAMTS4 in the presence of the metalloprotease inhibitor BB94 (C). In addition to the expected cleavage products Aβ-23-3 and Aβ4–16, further peptide fragments comprising Aβ-3-16, Aβ-23-(-4) and Aβ5–16 were observed upon incubation with ADAMTS4 (B), but not in the control conditions (A,C). Shown mass spectra are from a single experiment and correspond to reaction mixtures that were stopped by acidification immediately after mixing ($t = 0$ h; black traces) or after 4-h incubation ($t = 4$ h; red traces). The maximum of the individual isotope clusters is indicated by a dashed horizontal line to facilitate the comparison of signal intensities between the two time points. Full mass spectra of all experimental conditions are shown in Figure S2.

N-termini [23, 40, 41]. To enable quantitative inter-spectral comparison, Aβ signals were normalised to the signal of the internal spike Aβ5–38(D23S), which does not show mass overlap with any known Aβ species. In good agreement with our previously published data [13], Aβ4–40 peptide levels increased significantly by ~10-fold after induction of ADAMTS4 expression ($p < 0.0001$; Figure 3). Signals for Aβ4–x variants such as Aβ4–37, Aβ4–38, Aβ4–39 and Aβ4–42 were undetectable in noninduced cells, while considerable signal intensities were observed after ADAMTS4 induction (see list of reliably detected Aβ variants by our IP-MS approach in Table S2A,B), corroborating our previous finding that ADAMTS4 is critically involved in generating Aβ4–x peptide species [13]. Importantly, Aβ-3-40 was

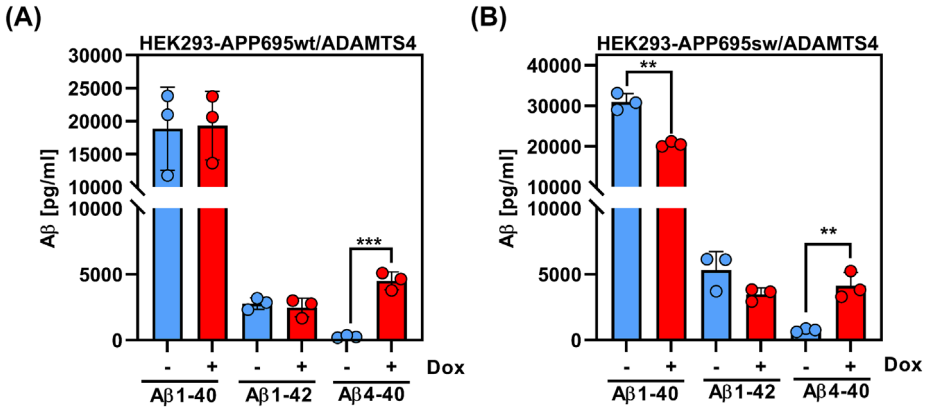

FIGURE 2 ELISA measurements of Aβ1–x and Aβ4–x peptides in conditioned culture supernatants of HEK293 cells with inducible overexpression of ADAMTS4. (A) In HEK293-APP695wt/ADAMTS4 cells, Aβ1–40 and Aβ1–42 levels were unchanged while Aβ4–40 levels were significantly increased after ADAMTS4 induction as compared with noninduced control cells. (B) Similarly, in HEK293-APP695sw/ADAMTS4 cells, induction of ADAMTS4 expression resulted in significantly increased Aβ4–40 levels, while Aβ1–40 levels were reduced. Three independent biological experiments for each cell line were performed ($n = 3$). DOX—doxycycline, $^{**}p < 0.01$, $^{***}p < 0.001$, mean ± SD, unpaired *t* test.

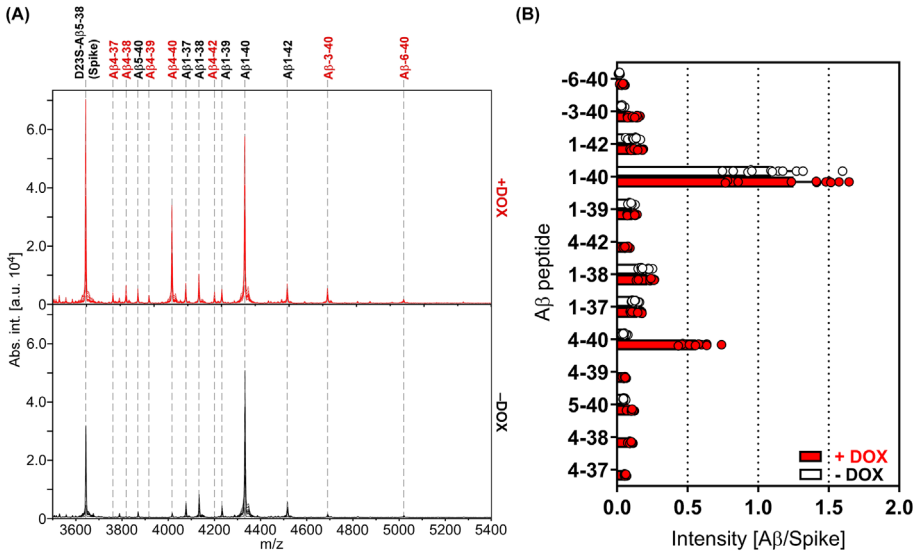

FIGURE 3 MALDI-TOF mass spectra of Aβ peptides immunoprecipitated from HEK293-APP695wt/ADAMTS4 cells. (A) Aliquots of conditioned cell culture media of HEK293-APP695wt/ADAMTS4 cells with (+DOX) and without (−DOX) induction of ADAMTS4 expression were subjected to immunoprecipitation with 6E10-coupled magnetic beads and analysed by MALDI-TOF-MS. The artificial model peptide Aβ5–38(D23S) was added as a spike for normalisation. An overlay of 12 linear mode mass spectra per condition is shown. Black and red traces correspond to samples without (−DOX) and with (+DOX) induction of ADAMTS4 expression, respectively. The annotation of Aβ signals was based on monoisotopic masses derived from reflector mode mass spectra (see Table S2A,B). (B) Quantitative comparison of the Aβ signals shown in (A). The ratio of the signal intensities of the Aβ peptide of interest divided by the signal intensity of the Aβ5–38(D23S) peptide used for normalisation was calculated. Significant differences in normalised signal intensities after induction of ADAMTS4 expression were observed for Aβ-3-40, Aβ-6-40, Aβ4–40 and Aβ5–40 peptides (mean ± SD, multiple *t* tests). Note that signals for Aβ4–37, Aβ4–38, Aβ4–39 and Aβ4–42 were undetectable in noninduced cells and thus not included in the statistical analysis. The 12 mass spectra per condition were obtained from three individual IPs, two MALDI preparations per IP, and two measurements per preparation.

detected with low abundance in noninduced HEK-APP695wt/ADAMTS4 cells but showed a significant ∼threefold increase in signal intensity after induction of ADAMTS4 expression ($p < 0.0001$). Interestingly, Aβ-6-40 was detected with a ∼twofold increase as a further N-terminally elongated Aβ variant ($p < 0.0001$; Figure 3). In addition, the small Aβ5–40 signal was increased by a factor of ∼2 ($p < 0.0001$).

We further sought to investigate which peptides can be immunoprecipitated from supernatants of HEK293-APP695wt/ADAMTS4 cells with the antibody 101-1-1 that was used as a capture antibody in our previously described Aβ-3-40 sandwich immunoassay [23]. After induction of ADAMTS4 expression, aside from the main signal representing Aβ-3-40 peptides already identified by IP-MS with 6E10,

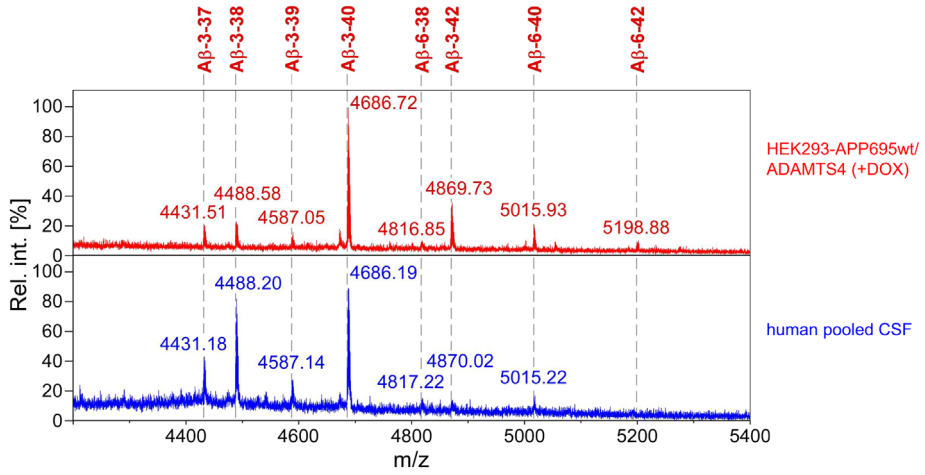

FIGURE 4 IP-MS characterisation of N-terminally elongated Aβ peptides in cell culture supernatants and pooled human CSF immunoprecipitated with mAb 101-1-1 (raised against Aβ-3-x peptides). Aliquots of conditioned cell culture media of HEK293-APP695wt/ADAMTS4 cells with (+DOX) induction of ADAMTS4 expression (upper panel) and pooled human CSF (lower panel) were subjected to immunoprecipitation with 101-1-1-coupled magnetic beads and analysed by MALDI-TOF-MS in reflector mode. After ADAMTS4 induction, a series of Aβ-3-x peptides with varying C-termini became detectable (Aβ-3-37/38/39/40/42). In addition, Aβ species with an even further elongated N-terminus starting with Ile-(-6) (Aβ-6-38/40/42) were detected. One mass spectrum out of two per condition is shown.

a series of smaller signals corresponding to Aβ-3-37, Aβ-3-38, Aβ-3-39 and Aβ-3-42 were detected (Figure 4; Figure S4). A series of peptides corresponding to Aβ-6-38, Aβ-6-40 and Aβ-6-42 was also identified, corroborating an additional ADAMTS4 cleavage site within the APP sequence between residues Glu-(-7) and Ile-(-6) at the -6-position N-terminal of the Aβ peptide sequence (Figure 4; Figure S4). In order to study whether these additional N-terminally elongated Aβ-6-x variants can be also identified in human CSF, pooled CSF samples were preconcentrated via centrifugal filtration and subjected to 101-1-1 immunoprecipitation and MALDI-TOF as described above. In agreement with the results obtained in the conditioned media, a series of Aβ-3-x peptides, as well as Aβ-6-38 and Aβ-6-40 were identified (Figure 4).

Next, to accurately quantify N-terminally elongated Aβ peptide variants in tissue culture supernatants, an in-house developed electrochemiluminescence immunoassay was used, which had been characterised and validated in a previous study [23]. In order to define a suitable dilution factor, conditioned supernatants from HEK293-APP695wt/ADAMTS4 and HEK293-APP695sw/ADAMTS4 cells with or without induction of ADAMTS4 expression were measured in twofold to 16-fold dilutions, leading to a gradual decrease in raw signal intensities with increasing sample dilution. Plotting the dilution-corrected Aβ-6/-3-40-concentrations against the dilution factor indicated that a fourfold to eightfold dilution allowed measurement of Aβ peptide signals in the assay detection range while avoiding potential matrix interference effects (Figure S3).

We then used this assay to measure the levels of N-terminally elongated Aβ levels in conditioned media of a variety of HEK293 cell lines. Stable overexpression of human APP695wt in HEK293-APP695wt cells led to a highly significant, approximately 10-fold increase in Aβ-6/-3-40 levels compared with nontransfected HEK293 cells (814 ± 70 pg/mL vs 75 ± 9 pg/mL respectively; $p < 0.0001$). In HEK293-APP695wt/ADAMTS4 cells, DOX induction of ADAMTS4 expression resulted in a further, approximately 2.6-fold increase in Aβ-6/-3-40 levels in comparison to noninduced cells (3962 ± 291 pg/mL vs 1552 ± 46 pg/mL; $p < 0.0001$). The slightly higher Aβ-6/-3-40 levels in noninduced HEK293-APP695wt/ADAMTS4 cells compared with the parental HEK293-APP695wt cells suggested some leakage of the ADAMTS4 inducible expression system even in the absence of DOX (Figure 5A). In contrast, neither overexpression of human APP695sw in HEK293-APP695sw cells nor induction of ADAMTS4 expression in HEK293-APP695sw/ADAMTS4 cells significantly altered the measurable Aβ-6/-3-40 levels compared with nontransfected HEK293 cells. This was expected, because mAb101-1-1, which was raised against the N-terminus of Aβ-3-x (corresponding to APP669–x), cross-reacts with the wild type APP sequence but not with APP carrying the Swedish 670/671 KM/NL double mutation, which is located directly adjacent to Val-(-3) of the Aβ(-3-40) sequence [23] (Figure 5A). Therefore, the low levels of N-terminally elongated Aβ species detected in both cell lines with stable overexpression of APP695sw likely resulted from the processing of endogenous, wild type APP.

In order to evaluate whether the observed increase in N-terminally elongated Aβ-6/-3-40 levels upon induction of ADAMTS4 expression might be cell-type dependent and restricted to the HEK293 cell line, additional mammalian cell lines were investigated. Very low Aβ-6/-3-40 levels were detected in conditioned media of parental H4 neuroglioma cells (61 ± 44 pg/mL). In H4 cells with stable overexpression of APP695wt, Aβ-6/-3-40 levels were considerably increased (720 ± 109 pg/mL, $p < 0.01$; Figure 5B). Likewise, we were unable to detect Aβ-6/-3-40 in nontransfected Chinese hamster ovary (CHO) cells, but Aβ-6/-3-40 levels were clearly

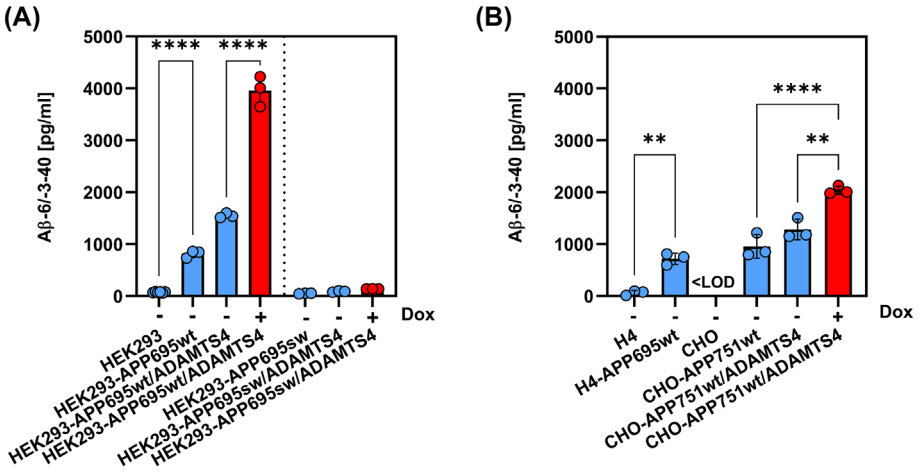

FIGURE 5 Comparison of Aβ-6/-3-40 peptide levels as determined with an electro-chemiluminescent assay in (A) conditioned media of nontransfected HEK293 cells, HEK293 cells with stable overexpression of either APP695wt or APP695sw, and HEK293 cells with stable overexpression of APP and doxycycline (DOX) inducible expression of ADAMTS4 ($n = 3$–6 experiments, one-way ANOVA). (B) Aβ-6/-3-40 peptide levels in conditioned media of nontransfected H4 and CHO cells, H4 and CHO cells with stable overexpression of wild type APP, and CHO cells with stable overexpression of wild type APP and doxycycline (DOX) inducible expression of ADAMTS4 ($n = 3$, mean ± SD, unpaired *t* test and one-way ANOVA).

measurable in CHO cells with stable overexpression of APP751wt (955 ± 229 pg/mL). Induction of ADAMTS4 expression in CHO-APP751wt/ADAMTS4 cells resulted in significantly increased Aβ-6/-3-40 levels vs noninduced cells (2039 ± 80 pg/mL, $p < 0.01$; Figure 5B), confirming the results obtained with HEK293-APP695wt/ADAMTS4 cells. Lastly, we wanted to verify that the signals measured by the 101-1-1 immunoassay in cells with stable overexpression of APP695wt but without co-expression of ADAMTS4 were indeed attributable to the presence of N-terminally elongated Aβ peptides (Figure 5A). Therefore, conditioned media of HEK293-APP695wt cells were subjected to a single round of magnetic bead immunoprecipitation with 6E10 and subsequent analysis by MALDI-TOF-MS as described above. While no signals were obtained for nontransfected cells, Aβ-3-40 was detected in the conditioned media of HEK293-APP695wt but not HEK293-APP695sw cells. However, we obtained clear signals for Aβ1–42 in the conditioned media of HEK293-APP695sw cells, with the expected increased signal intensity as compared with parental HEK293 cells due to the overexpression of APP (Figure S5). Quantitative real-time PCR measurements of ADAMTS4 expression in HEK293 and H4 cells revealed only negligible expression levels, raising the possibility that other proteases might participate in the generation of Aβ-3-x peptides (Figure S6).

Extracellular and vascular amyloid deposits in some AD patients are immunoreactive for Aβ-6/-3-x peptides

Up to this point, the potential presence of N-terminally elongated Aβ peptides in human brain tissue had not been investigated. Therefore, we further characterised the binding epitopes of the mAbs 101-1-1 and 14-2-4 (both raised against Aβ-3-x [23]) with peptide microarrays containing overlapping 15-mer Aβ peptides ranging from position −23 to 51 (aka APP649–722). This analysis showed recognition of overlapping N-terminal peptides extending from Lys-(−10) to His-(13) and revealed the minimal contact motif as Aβ-2-5 (KMDAEFR) (Figure 6A,B). A peptide preadsorption experiment in human AD brain tissue showed that 101-1-1 immunoreactivity was effectively blocked by preadsorption of the antibody with excess amounts of synthetic Aβ-3-40 but not Aβ1–40 peptides, which lacked the crucial "KM" residues from the core motif (Figure 6C–F′). We then employed mAbs 101-1-1 and 14-2-4 for immunohistochemistry on a series of human brain samples from sporadic AD, Down syndrome (DS) and nondemented control (NDC) cases. For comparison, the Aβ antibody 24311 as well as the Aβ4–x-specific antibody 18H6 were used. A semiquantitative analysis revealed that the majority of the sporadic AD, all DS and some of the nondemented control cases demonstrated a high abundance of extracellular amyloid deposits detected with both 24311 and 18H6 (Figure 7A–D). While nondemented control cases were consistently negative (Figure S7), considerable immunoreactivity with the antibodies 101-1-1 and 14-2-4 was detected in four out of 30 sporadic AD cases (13%), as well as in one individual with DS (Figure 7E). A comparative analysis in consecutive sections revealed N-terminally elongated Aβ immunoreactivity in the same deposits as those labelled with 24311 and 18H6, albeit with reduced staining intensity (Figure 7A′–D′).

Finally, we carried out a comparative analysis of parenchymal vessel pathology, which was, in general, less obvious than extracellular Aβ immunoreactivity. Fewer cases showed parenchymal vascular Aβ immunoreactivity compared with extracellular plaque pathology. Again, positive vascular 101-1-1 and 14-2-4 staining was observed only in a subset of samples, including all five cases showing 101-1-1 and 14-2-4 immunoreactivity in extracellular deposits (Figure 8). Because we had previously shown that antibody 101-1-1 is able to detect human wild type APP and recombinant sAPPα [23], we stained adjacent sections

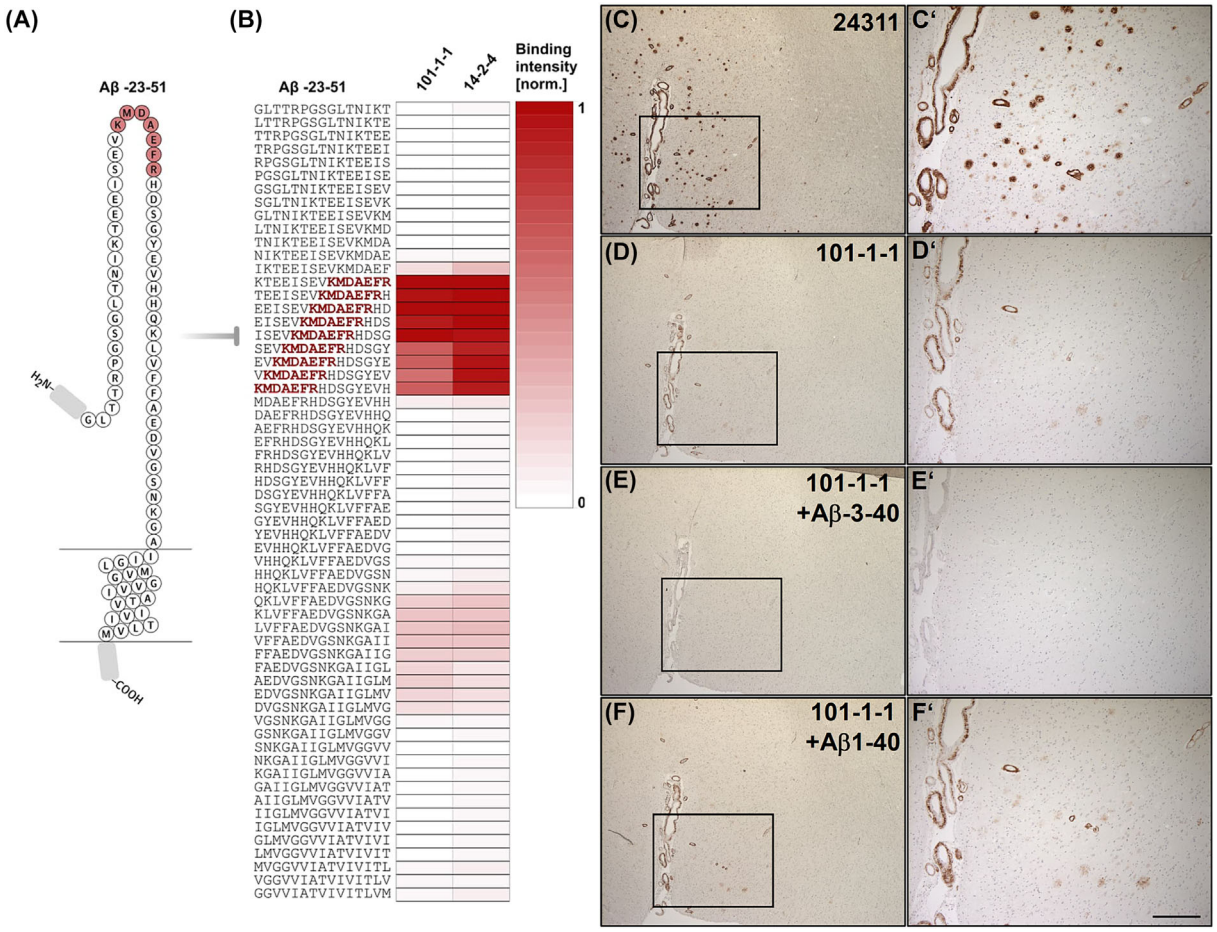

FIGURE 6 Peptide microarray screening of Aβ-23-51 against monoclonal antibodies 101-1-1 and 14-2-4 defined a common core motif. (A), pictogram of the selected Aβ-23-51 sequence for microarray analysis, the core motif has been highlighted in red. (B), the Aβ-23-51 sequence was presented as overlapping 15-meric peptides shifted by one residue. Binding of antibodies 101-1-1 and 14-2-4 was detected with goat anti-mouse HRP secondary antibody, both peptide signals were internally normalised and depicted as a heat map on a red scale [0-1], where 1 corresponds to the highest detected binding. Parallel sections from an AD patient were stained with 24311 (C,C′), 101-1-1 (D,D′), 101-1-1 preadsorbed with Aβ-3-40 (E,E′) and 101-1-1 preadsorbed with Aβ1–40 (F,F′). Scale bars: A–D = 500 μm; A′–D′ = 200 μm.

with the APP N-terminus-specific antibody 22C11 or with antibodies IG7/5A3 raised against a central APP epitope to evaluate whether the observed 101-1-1 or 14-2-4 immunoreactivity was due to the detection of full-length APP or N-terminal APP fragments. While in some cases a 101-1-1 staining pattern was observed that resembled 22C11 or IG7/5A3 immunoreactivity in dystrophic neurites of Aβ deposits (Figure S8), other cases showed an entirely different pattern, with a lack of labelling of APP-positive dystrophic neurites and the presence of a prominent plaque core immunoreactivity (Figure 9). Importantly, 22C11 and IG7/5A3 showed an entirely different and nonoverlapping staining pattern compared with 101-1-1 or 14-2-4 in the vasculature (Figure S8). The vascular staining with 101-1-1 was also completely abolished following preadsorption with synthetic Aβ-3-40 peptides.

DISCUSSION

The enzymatic machinery that generates Aβ peptides from APP is a key target for the development of disease-modifying therapies to treat or prevent AD. BACE1 is the main protease responsible for the initial "β-site" cleavage of APP and generates the N-terminus of the canonical Aβ1–x sequence [4]. However, several other proteases such as aminopeptidase A, meprin-β or neprilysin are capable of cleaving APP close to the BACE1 site, thereby generating N-terminally truncated Aβ peptide variants [42–44]. Recently, we have shown that the metalloprotease ADAMTS4 generates Aβ4–x species in vitro and in vivo [13], which are highly abundant Aβ species found in human AD brains [6]. This enzyme is a member of the aggrecan-degrading ADAMTS family and is exclusively expressed in oligodendrocytes in the murine brain [13, 14, 45, 46]. In this study, we demonstrated the presence of another ADAMTS4 cleavage site N-terminal to the BACE1 site between residues Glu-(-4) and Val-(-3), resulting in the release of N-terminally elongated Aβ-3-x peptides. Importantly, Aβ-3-40 (aka. APP669–711) has been used as a reference peptide in blood plasma to normalise the large interindividual variances in the levels of the diagnostically relevant Aβx–42 species [20, 21]. Furthermore, IP-MS determination of a composite biomarker consisting of the Aβ-3-40/Aβ1–42 and Aβ1–40/Aβ1–42 ratios has been shown to

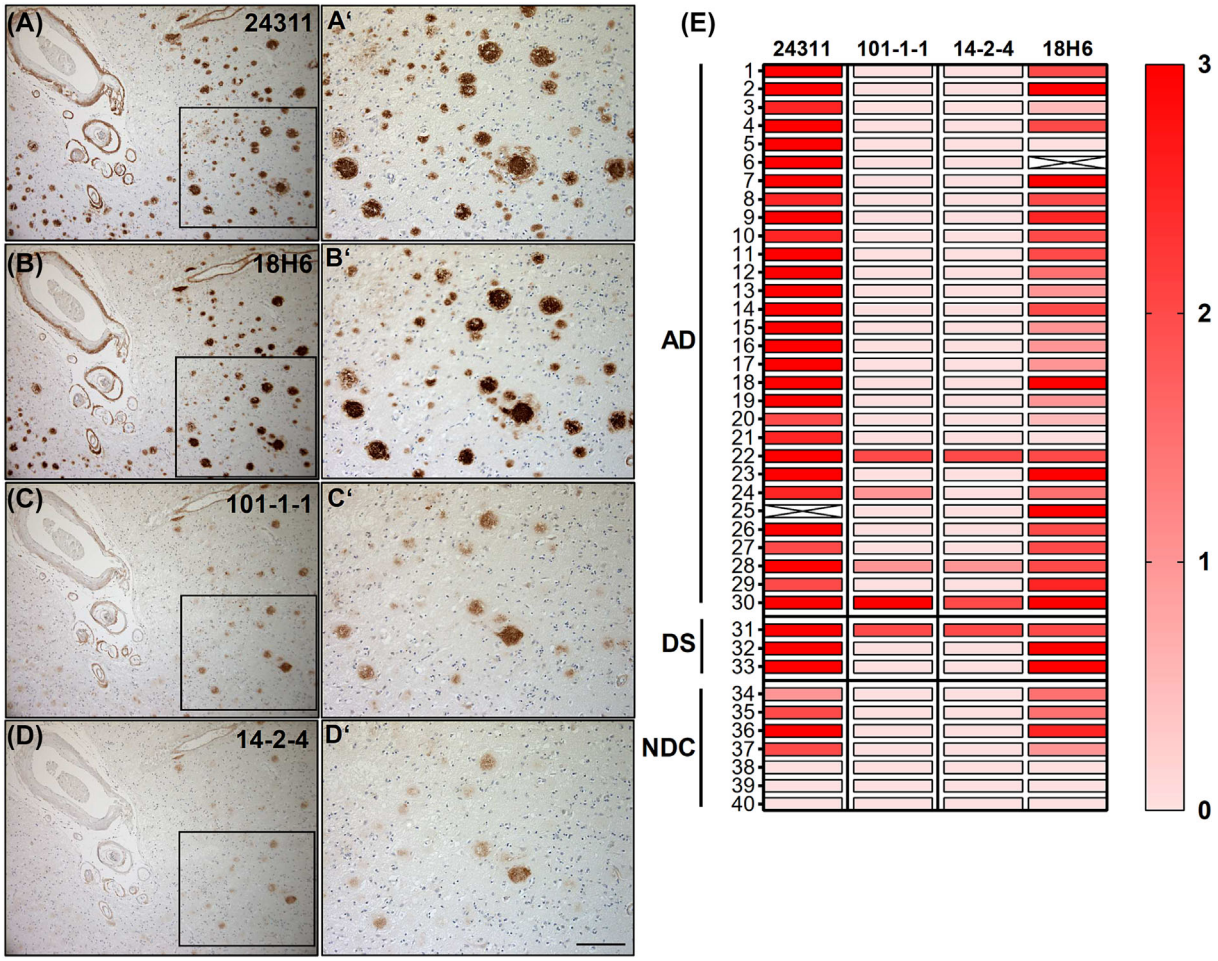

FIGURE 7 Immunohistochemical staining of extracellular Aβ peptides in a sporadic Alzheimer's disease case (AD case 30). Parallel sections of the medial frontal gyrus were stained with the Aβ antibody 24311 (A,A′), the Aβ4–x-specific antibody 18H6 (B,B′), as well as antibodies 101-1-1 (C,C′) and 14-2-4 (D,D′) detecting N-terminally elongated Aβ peptides. (E) Heat map illustrating the semiquantitative analysis of the immunostainings of parenchymal plaques. Scale bar: A–D: 200 μm, A′–D′: 100 μm.

accurately predict brain Aβ accumulation in amyloid-β PET positive individuals, promising a plasma biomarker with clinical utility at an individual level [20]. However, the proteolytic mechanism through which Aβ-3-40 peptides are produced has remained unresolved. Our findings that ADAMTS4 is the responsible protease are largely consistent with a recent study by Matsuzaki and colleagues [22], and in aggregate, both studies strongly indicate that ADAMTS4 is involved in the generation of N-terminally elongated Aβ-3-x peptides in vivo.

Previously, we had established that full-length Aβ1–x peptides can serve as direct substrates for recombinant ADAMTS4 in vitro [13]. Therefore, in order to investigate whether ADAMTS4 could generate N-terminally elongated Aβ-3-x peptide variants, we incubated the synthetic Aβ-23-16 model peptide (APP649–687) [23] with recombinant ADAMTS4. Using MALDI-TOF MS, we were able to detect the cleavage products Aβ-23-(-4) and Aβ-3-16 providing evidence that ADAMTS4 can indeed cleave between residues Glu-(-4) and Val-(-3) three amino acids N-terminal to the BACE1 cleavage site. Using a larger recombinant substrate protein corresponding to APP619–699, Matsuzaki and colleagues obtained similar results and showed that ADAMTS4 was capable of cleaving at three sites N-terminal to the amino acids Val-(-3), Glu-(4) and Val-(12) of the Aβ sequence [22].

To confirm these findings in a more relevant cellular context, we made use of HEK293 and CHO cell lines with stable overexpression of wild type APP695 and DOX-inducible expression of ADAMTS4 and previously developed electrochemiluminescence and IP-MS based assays for the detection of Aβ-3-x peptides [19, 23]. Depending on the cell line and the detection method, a twofold to threefold increase in secreted N-terminally elongated Aβ peptides was observed after induction of ADAMTS4 expression. This indicated ADAMTS4-dependent generation of Aβ-3-x peptides in line with findings by Matsuzaki and colleagues in HEK293 cells transiently transfected with APP and ADAMTS4 [22]. IP-MS analysis with antibody 101-1-1, which was raised against a peptide comprising the first five amino acids of the Aβ sequence and the three amino acids N-terminal of the Asp in position 1 (VKMDAEFR) [23], further identified a series of C-terminal variants of Aβ-3-x peptides, ranging from Aβ-3-37 to Aβ-3-42 after induction of ADAMTS4 expression. Interestingly, additional N-terminally elongated Aβ peptide variants starting with isoleucine at the -6-position (Aβ-6-38, Aβ-6-40, Aβ-6-42) were also detected in cell

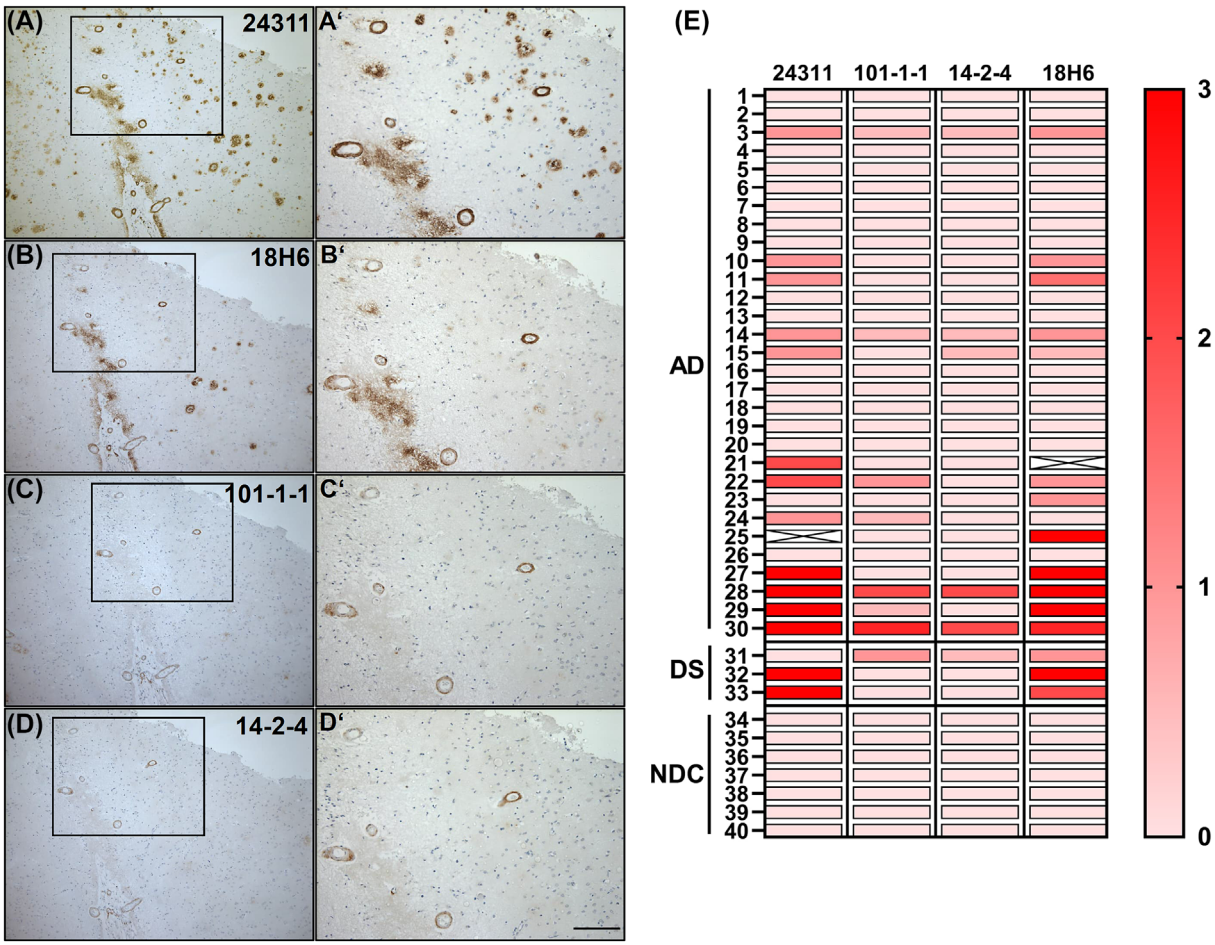

FIGURE 8 Immunohistochemical staining of vascular Aβ deposits in a sporadic Alzheimer's disease case (AD case 28). Parallel sections of the medial frontal gyrus were stained with the Aβ antibody 24311 (A,A′), the Aβ4–x-specific antibody 18H6 (B,B′), as well as antibodies 101-1-1 (C,C′) and 14-2-4 (D,D′) detecting N-terminally elongated Aβ peptides. (E) Heat map illustrating the semiquantitative analysis of the immunostainings of parenchymal vascular amyloid angiopathy. Scale bar: A–D: 200 μm, A′–D′: 100 μm.

culture supernatants as well as in human pooled CSF, which had not been described in the study by Matsuzaki and colleagues [22]. In support of these Aβ-6-x peptides occurring under physiological conditions, Aβ-6-38 and Aβ-6-40 had been previously reported in human plasma samples by IP-MS and were suggested to originate from a proteolytic pathway independent of α- and β-secretase [18].

One issue arising from our studies is whether ADAMTS4 is the only protease responsible for the generation of the described N-terminally elongated Aβ peptides. In HEK293, H4 and CHO cells with stable overexpression of APP695wt, substantial amounts of Aβ-6/-3-40 peptides were detectable with our electrochemiluminescence assay even without co-expression of ADAMTS4. In fact, measurable Aβ-6/-3-40 signals above the lower limit of detection were observed in conditioned media of nontransfected H4 and HEK293 cells. In a previous study [22], Aβ-3-40 was not detected in nontransfected H4 cells, but this difference might be explained by the higher sensitivity of the electrochemiluminescence detection method used here compared with the IP-MS approach. Through IP-MS analysis, we confirmed the presence of Aβ-3-40 peptides in conditioned media of HEK293 cells with stable overexpression of APP695wt, supporting the specificity of the electrochemiluminescence assay. However, qPCR analysis did not provide convincing evidence for endogenous ADAMTS4 expression in HEK293 or H4 cells, indicating that Aβ-3-40 peptides in the cell lines without ectopic ADAMTS4 expression were likely generated by another protease. This conclusion was also supported by Matsuzaki and colleagues [22] through genome editing experiments in nontransfected A549 adenocarcinoma cells, which secreted detectable levels of Aβ-3-40 peptides. CRISPR/Cas9 genome editing of the ADAMTS4 locus in A549 cells reduced secreted Aβ-3-40 levels, but only by around 30%–40%. While A549 cells contain multiple copies of the ADAMTS4 gene complicating the genomic confirmation of a complete gene knockout, these authors also reported a significant but less than 50% reduction in Aβ-3-40 plasma levels in ADAMTS4 knockout mice [22]. Hence further studies are clearly required to clarify whether the generation of Aβ-3-x peptides in humans is mainly dependent on the protease activity of ADAMTS4.

The presence of Aβ-3-40 peptides in human plasma [18, 20, 21, 23] and CSF [19] has been previously demonstrated. Earlier studies have also characterised the biophysical properties of synthetic N-terminally elongated Aβ peptides (Aβ-3-38, Aβ-3-40, Aβ-3-42) and

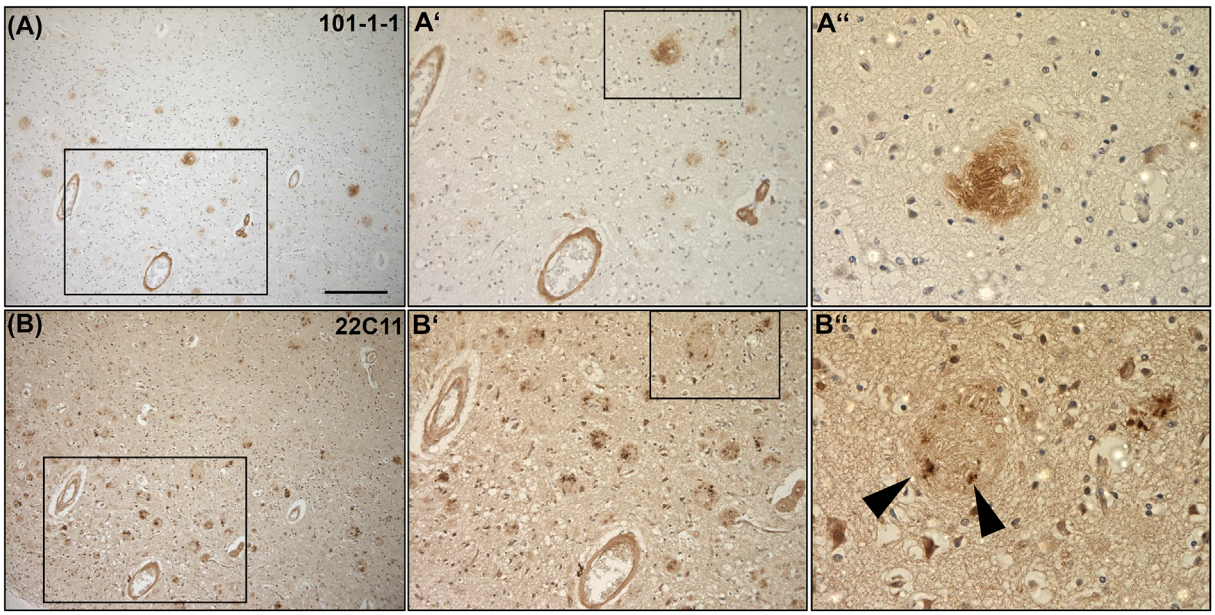

FIGURE 9 Immunohistochemical staining of parallel sections (case AD 30) with 101-1-1 (A–A″) and the N-terminal APP antibody 22C11 (B–B″). While 101-1-1 stains plaque cores, 22C11 decorates dystrophic neurites (arrowheads in B″). Scale bar: A,B = 200 μm; A′,B′ = 100 μm; A″,B″ = 33 μm.

have revealed that all three peptide variants are capable of aggregation into thioflavin T-positive amyloid fibrils [47], although Aβ-3-40 showed a lower tendency to self-assembly compared with Aβ1–42 [20]. In addition, evidence has been provided for the co-aggregation of endogenous murine Aβ-3-40 peptides with human Aβ in the brains of APP-transgenic mice with plaque pathology [22]. However, information about the occurrence and localization of Aβ-3-x peptides in the human brain is lacking. Therefore, we carried out an immunohistochemical analysis in paraffin-embedded brain samples from individuals suffering from AD and DS as well as nondemented control (NDC) cases. We employed a set of carefully characterised antibodies detecting Aβ-3-x [23], total Aβ or Aβ4–x species [48]. In good agreement with a previous study [48], the Aβ antibody 24311 and the Aβ4–x-selective antibody 18H6 stained extracellular parenchymal plaques in all AD and DS cases as well as in a subset of the NDC samples. With the antibodies detecting Aβ-3-x peptides, a mild to moderate staining of extracellular plaques was only observed in a subset of disease cases (13%) but in none of the NDC samples. Similar results were obtained with regard to Aβ immunoreactivity within the vasculature, with Aβ-3-x immunoreactivity mainly present in those cases showing also extracellular Aβ-3-x plaque pathology. Preadsorption of 101-1-1 with synthetic Aβ-3-40 but not Aβ1–40 peptides completely abolished 101-1-1 immunoreactivity, supporting the assumption that Aβ-3-x peptides indeed represented the main target in 101-1-1 positive human brain samples. Furthermore, microarray analysis against Aβ-23-51 overlapping peptides with antibodies 101-1-1 and 14-2-4 showed a consistent shared motif (KMDAEFR), in agreement with the employed immunogen sequence. This further clarified the lack of recognition of the Swedish double mutation, as the core motif does not allow the exchange of Lys-670. As these antibodies also detect wild type APP in Western-blotting assays [23], parallel sections were stained with the 22C11 and IG7/5A3 antibodies detecting N-terminal or central APP epitopes. In most cases, an entirely different staining pattern was observed with regard to parenchymal deposits, indicating that major cross-reactivity of the Aβ-3-x detecting antibodies with APP was unlikely. While 101-1-1 showed strong immunoreactivity in amyloid plaque cores, 22C11 or IG7/5A3 only decorated dystrophic neurites in the vicinity of plaques. In line with these findings, the same staining pattern with decorated dystrophic neurites has previously been described for antibodies detecting Aη epitopes, which are located close to the Aβ peptide sequence in APP [49] and which seem to be generated in part by MT5-MMP, a membrane-bound matrix metalloproteinase [50]. After shedding of the APP ectodomain by MT5-MMP, C-terminal CTFη fragments are further processed by ADAM10 and BACE1 resulting in the generation of Aη-α and Aη-β peptides [49]. While detection of Aη-α by our antibodies cannot be ruled out completely, detection of Aη-β is unlikely as the core motif needed for recognition has been determined as Aβ-2-5. Importantly, no overlap between 101-1-1 or 14-2-4 staining with APP-specific antibodies was observed in the vasculature. These findings, together with the earlier identification of Aβ-3-40 peptides in human CSF [19], suggest that Aβ-3-x peptide species deposit within the brain parenchyma in extracellular plaques or in the vasculature under certain conditions. Average levels of Aβ-3-40 in plasma [20] and CSF [19] samples from amyloid-β-PET positive and negative individuals were comparable. However, some individuals displayed substantially higher or lower Aβ-3-40 plasma levels [20], and whether this is related to brain deposition of Aβ-3-40 remains to be investigated. Alternatively, differential expression of the proteolytic enzymes involved in the generation of Aβ-3-x peptides could explain inter-individual variations in Aβ-3-40 levels. Whether ADAMTS4 expression in the brain oligodendrocyte population is regulated is not

known. However, in the periphery, ADAMTS4 expression can be upregulated by pro-inflammatory cytokines, raising the possibility that Aβ-3-40 plasma levels might be affected by inflammatory conditions [51]. In summary, we have now demonstrated that ADAMTS4 facilitates the generation of N-terminally truncated Aβ4–x and N-terminally elongated Aβ-6/-3-x peptides, potentially implicating this secreted metalloprotease in both the pathological process of Aβ aggregation and the early detection of the amyloid pathology in AD.

AUTHOR CONTRIBUTIONS

Oliver Wirths designed the study, performed experiments and drafted the manuscript; Christina Lehnen, Merle Fricke, Ivan Talucci, Barbara Morgado, Sandra Lehmann, Carolina Münch and Thomas Liepold performed experiments and acquired data; Hans-Wolfgang Klafki, Olaf Jahn, Hans Michael Maric, Oliver Wirths and Sascha Weggen analysed data and revised the manuscript. Jorge Ghiso and Agueda Rostagno provided reagents and Jens Wiltfang provided reagents and the Aβ-3-40 immunoassay platform. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The datasets used and/or analysed in the present study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All procedures involving human tissue and archived CSF samples have been approved by the ethical committee at the University Medical Center Göttingen (Protocol number 12/1/15; 9/2/16). All tissues have been received from the Netherlands Brain Bank (NBB) and all material and data collected by the NBB are obtained based on written informed consent.

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