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ORIGINAL PAPER

Abeta targets of the biosimilar antibodies of Bapineuzumab, Crenezumab, Solanezumab in comparison to an antibody against N-truncated Abeta in sporadic Alzheimer disease cases and mouse models

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Abstract Solanezumab and Crenezumab are two humanized antibodies targeting Amyloid-β (Aβ) which are currently tested in multiple clinical trials for the prevention of Alzheimer's disease. However, there is a scientific discussion ongoing about the target engagement of these antibodies. Here, we report the immunohistochemical staining profiles of biosimilar antibodies of Solanezumab, Crenezumab and Bapineuzumab in human formalin-fixed, paraffin-embedded tissue and human fresh frozen tissue. Furthermore, we performed a direct comparative immunohistochemistry analysis of the biosimilar versions of the humanized antibodies in different mouse models including 5XFAD, Tg4-42, TBA42, APP/PS1KI, 3xTg. The staining pattern with these humanized antibodies revealed a surprisingly similar profile. All three antibodies detected plaques, cerebral amyloid angiopathy and intraneuronal Aβ in a similar fashion. Remarkably, Solanezumab showed a strong binding affinity to plaques. We also reaffirmed that Bapineuzumab does not recognize N-truncated or modified Aβ, while Solanezumab and Crenezumab do detect N-terminally modified Aβ peptides Aβ4–42 and pyroglutamate Aβ3–42. In addition, we compared the results with the staining pattern of the mouse NT4X antibody that recognizes specifically Aβ4–42 and pyroglutamate Aβ3–42, but not full-length Aβ1–42. In contrast to the biosimilar antibodies of Solanezumab, Crenezumab and Bapineuzumab, the murine NT4X antibody shows a unique target engagement. NT4X does barely cross-react with amyloid plaques in human tissue. It does, however, detect cerebral amyloid angiopathy in human tissue. In Alzheimer mouse models, NT4X detects intraneuronal Aβ and plaques comparable to the humanized antibodies. In conclusion, the biosimilar antibodies Solanezumab, Crenezumab and Bapineuzumab strongly react with amyloid plaques, which are in contrast to the NT4X antibody that hardly recognizes plaques in human tissue. Therefore, NT4X is the first of a new class of therapeutic antibodies.

Keywords Alzheimer's disease · Immunotherapy · Abeta · Plaques · Congophilic amyloid angiopathy · Immunization

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Introduction

The amyloid-β hypothesis has been the most influential hypothesis for Alzheimer's disease (AD) and an important basis for the development of novel therapeutic strategies. The hypothesis suggests that increased Aβ production or decreased Aβ clearance leads to accumulation of hydrophobic $A\beta_{40}$ and $A\beta_{42}$ and the formation of insoluble extracellular plaques [29]. Plaques then trigger a cascade of toxic changes eventually resulting in synapse loss, neuron loss, brain atrophy and dementia [53]. If the amyloid-β hypothesis is indeed correct, intervening in the cascade and

removing Aβ from the brain should prevent neuron loss and cognitive decline. The main therapeutic intervention strategies directed at Aβ include reducing Aβ production, assisting Aβ clearance and preventing the aggregation of Aβ [55]. Active and passive immunotherapy procedures are considered to be among the most promising approaches to prevent or treat AD and over the last decade various anti-Aβ antibodies have been developed [24, 55].

Bapineuzumab was the first antibody used in a passive immunotherapy clinical trial. The humanized antibody developed by Pfizer and Johnson & Johnson targets the N-terminal region of Aβ binding fibrillar and soluble Aβ [68]. Bapineuzumab is the only antibody reported to clinically decrease the brain amyloid burden as well as reduce Tau and phosphorylated-Tau levels in the CSF in mild to moderate AD patients [9, 59]. However, large-scale phase three clinical trials were discontinued in 2012 as treatment evoked vasogenic edema and did not improve measures of cognitive decline in mild or moderate AD patients relative to placebo controls [9, 60, 69].

Several passive immunotherapy trials are currently ongoing [1]. Among them, Crenezumab (Genentech) and Solanezumab (Eli Lilly) are the leading clinical Aβ antibodies tested in multiple Phase II or III clinical trials [1]. These humanized monoclonal antibodies are highly homologous and target the mid-region of Aβ [14, 71]. Solanezumab is reported to bind preferentially to soluble monomers rather than fibrillar Aβ [65, 71]. Early clinical trials with Solanezumab showed increased CSF Aβ levels and large Phase 3 clinical trials reported that treatment with Solanezumab slowed down the cognitive decline in mild AD, but not in moderate AD patients [19, 68]. Crenezumab has a binding affinity to Aβ fibrils, oligomers and monomers [2]. The antibody, which unlike Solanezumab and Bapineuzumab is built on an IgG4 backbone, is currently being tested in several Phase II trials.

Several pre-clinical studies have demonstrated beneficial effects of passive immunotherapy against other therapeutic Aβ targets. N-truncated and modified Aβ species are highly represented in the earliest stages of AD as well as in fully developed AD and may, therefore, be potential therapy targets. For example, anti-pyroglutamate $Aβ_{3–42}$ antibodies reduced the plaque burden in AD mice, in the absence of microhemorrhage or increased vascular amyloid [17, 22, 75]. $Aβ_{4–42}$ is another abundant species in AD that is as toxic as $Aβ_{pE3–42}$ and $Aβ_{1–42}$ may, therefore, be a potential target for immunotherapy approaches [7].

In the present study, we performed a direct comparative immunohistochemistry analysis of the biosimilar versions of the humanized antibodies Bapineuzumab, Crenezumab and Solanezumab. The aim of the study was to investigate the binding profiles of these antibodies in different mouse models as well as in human tissue and compare the humanized antibodies with NT4X, an antibody that binds to the N-terminus of N-truncated Aβ especially $Aβ_{4–x}$.

Materials and methods

Human specimens

The brains of 35 sporadic AD patients (SAD; 32 females/3 males, mean age; mean age ± SEM 81.4 ± 1.58; Braak stage 4–6; ApoE4: 17/35) and 5 non-demented control patients (1 female/4 males, mean age ± SEM 81 ± 4.65; Braak stage 0–1) were analyzed (Table 1). All human cortical brain samples were obtained from the Netherlands Brain Bank (NBB, Amsterdam, The Netherlands) [56]. NBB works with a rapid autopsy program to minimize postmortem delay (PMD). Definite diagnosis was based on established criteria and written informed consent was obtained from all subjects. The control subjects did not suffer from any primary neurological or psychiatric disease or brain metastases nor did they have a history of medication or drug treatment. The inclusion as a control donor is based both on review of medical records and on the Braak stage by the Netherlands Brain Bank. All sporadic AD patients had Reisberg Scales of four or higher. The Reisberg Scale is used by caregivers as clinical diagnostic criteria and divided into seven different stages. Stages 4–7 are dementia stages, while stages 1–3 are pre-dementia stages [57].

Animal models

In this study, five established AD-like transgenic mouse lines (Table 2) were used: Tg4-42 [10], TBA42 [77], 5XFAD [48], APP/PS1KI [12] and 3xTg [49].

Tg4-42 mice express human $Aβ_{4–42}$ fused to the murine TRH signal peptide under the control of the neuronal Thy-1 promoter [10]. *TBA42* mice express $Aβ_{3Q–42}$ fused to the murine TRH signal peptide under the control of the neuronal Thy-1 promoter. The glutamate at position three of the Aβ amino acid sequence is mutated into glutamine to facilitate enhanced pyroglutamate formation. These mice express unmodified $Aβ_{3Q–42}$, which can be readily converted to $Aβ_{pE3–42}$ by glutaminyl cyclase [43, 77]. *5XFAD* mice overexpress the 695 amino acid isoform of the human amyloid precursor protein (APP695) carrying the Swedish, London and Florida mutations under the control of the murine Thy1-promoter. In addition, human presenilin-1 (PSEN-1) carrying the M146L/L286V mutations is expressed under the control of the murine Thy1-promoter [48]. *5XFAD* mice used in the current study were backcrossed for more than ten generations to C57Bl/6J wild-type mice (Jackson Laboratories, Bar Harbor, ME, USA) to obtain an incipient congenic line on a C57Bl/6J

Table 1 Clinical and pathological data of sporadic Alzheimer disease cases and controls

Case	Gender	Age of first symptoms	Age of death	Reisberg Score	Braak	ApoE	Post mortem delay
Sporadic AD cases							
AD-1	F	72	79	6	4B	43	05:20:00
AD-2	F	76	81	n.a.	4C	n.a.	04:50:00
AD-3	F	80	84	7	4B	32	04:15:00
AD-4	M	77	86	5	4C	n.a.	06:00:00
AD-5	F	76	86	6	4C	43	05:05:00
AD-6	F	84	88	7	4C	33	03:30:00
AD-7	F	88	88	7	4C	33	12:15:00
AD-8	F	81	88	6	4C	n.a.	05:25:00
AD-9	F	84	91	5	4C	43	03:45:00
AD-10	M	83	91	6	4C	n.a.	04:10:00
AD-11	F	89	92	4	4C	32	04:15:00
AD-12	M	84	93	7	4C	n.a.	05:50:00
AD-13	F	86	94	6	4C	32	04:20:00
AD-14	F	n.a.	68	n.a.	5C	33	04:51:00
AD-15	F	65	69	6	5C	42	07:00:00
AD-16	F	n.a.	78	7	5C	43	04:00:00
AD-17	F	74	78	7	5C	33	08:15:00
AD-18	F	68	78	5	5C	44	04:50:00
AD-19	F	73	79	7	5C	33	04:15:00
AD-20	F	67	82	6	5C	43	04:35:00
AD-21	F	73	83	7	5C	43	07:17:00
AD-22	F	81	84	7	5C	33	05:15:00
AD-23	F	67	84	n.a.	5C	43	06:30:00
AD-24	F	78	84	6	5C	33	05:55:00
AD-25	F	78	85	7	5C	43	06:10:00
AD-26	F	70	85	7	5C	44	05:00:00
AD-27	F	79	87	7	5C	33	09:15:00
AD-28	F	80	88	7	5C	33	03:15:00
AD-29	F	83	90	5	5B	42	04:30:00
AD-30	F	49	58	7	6C	43	04:30:00
AD-31	F	53	62	7	6C	43	04:45:00
AD-32	F	49	65	7	6C	33	05:40:00
AD-33	F	55	65	7	6C	33	06:20:00
AD-34	F	53	70	7	6C	33	08:18:00
AD-35	F	86	86	7	6C	33	03:40:00
Non-demented control cases							
Control-1	M	–	91	n.a.	I0	n.a.	<39:20:00
Control-2	F	–	90	n.a.	I0	n.a.	07:15:00
Control-3	M	–	84	n.a.	I0	n.a.	09:00:00
Control-4	M	–	70	n.a.	0/0	n.a.	07:30:00
Control-5	M	–	70	n.a.	0B	n.a.	07:45:00

n.a. not available

genetic background [32]. *3xTg* mice (a generous gift by Dr. Wolfgang Härtig, University of Leipzig) represent a triple-transgenic model of AD harboring three mutated transgenes: PS1-M146V, APPSwe, and tauP301L [49]. The *APP/PS1KI* transgenic mouse model (a generous gift by Dr. Laurent Pradier, Sanofi, Paris) carries M233T/L235P knocked-in mutations in presenilin-1 and overexpresses mutated human amyloid precursor protein carrying the

London and Swedish mutations under the Thy-1 promoter [12]. APP/PS1KI mice were backcrossed for more than 10 generations on a C57BL/6J genetic background.

All animal experiments were conducted in accordance with the German guidelines for animal care and approved by the local legal authorities (LAVES).

Immunohistochemistry

Mice were killed via CO_2 anesthetization followed by cervical dislocation. Brain samples were carefully dissected and post-fixed in 4 % phosphate-buffered formalin at 4 °C.

Human and mouse tissue samples were processed as previously described [10, 73]. In brief, 4 µm paraffin sections were deparaffinized in xylene, followed by rehydration in a series of ethanol. After H_2O_2 treatment to block endogenous peroxidases, sections were boiled in 0.01 M citrate buffer for antigen retrieval, followed by a 3-min incubation in 88 % formic acid. Non-specific binding sites were blocked by treatment with skim milk and fetal calf serum in PBS, prior to the addition of the primary antibodies. The following biosimilar antibodies (BS) were used (Table 3): $Bapineuzumab_{BS}$ (1:2500; 0.7 mg/ml), $Crenezumab_{BS}$ (1:2500; 0.85 mg/ml), $Solanezumab_{BS}$ (1:2500; 1.5 mg/ml) and NT4X (1:200; 2 mg/ml) [4, 14, 44]. Corresponding biotinylated secondary anti-human and anti-mouse antibodies (1:200) were purchased from DAKO (Glostrup, Denmark). Staining was visualized using the ABC method, with a Vectastain kit (Vector Laboratories, Burlingame, USA) and diaminobenzidine (DAB) as chromogen. Counterstaining was carried out with hematoxylin.

In addition to formalin-fixed, paraffin-embedded tissue fresh frozen cortical human brains and mouse brain hemispheres were embedded in Tissue-Tec (Sakura, Torrance, USA) and cut sagittal into entire series of 10 µm thick sections on a CM1850 UV cryostat (Leica, Wetzlar, Germany). Brains were mounted onto superfrost slides and fixed in −20 °C methanol. After hydration in PBS and H_2O_2 treatment to block endogenous peroxidases, sections were washed and incubated in 88 % formic acid for 3 min. Non-specific binding sites were blocked by treatment with skim milk and fetal calf serum in PBS. Prior to the addition of the antibodies the human antibodies $Bapineuzumab_{BS}$, $Crenezumab_{BS}$ and $Solanezumab_{BS}$ were biotinylated with a Biotinylation kit (EZ-Link Sulfo-NHS-LC-Biotinylation Kit) according to the instruction of the manufacturer (Thermo Scientific, Waltham, USA). Section was incubated over-night with biotinylated $Bapineuzumab_{BS}$, $Crenezumab_{BS}$ and $Solanezumab_{BS}$ as well as NT4X. Staining was visualized using the ABC method, with a Vectastain kit (Vector Laboratories, Burlingame, USA) and diaminobenzidine (DAB) as chromogen. Counterstaining was carried out with hematoxylin.

Table 2 Transgenic AD mouse models used

Mouse model	Promoter	AβPP	PS1	Tau	Age tested	Plaque onset (m)	Intraneuronal Aβ	References
5XFAD	Thy1 (APP, PS1)	Swe, Flo, Lon	M146L, L286V	–	6w (hom), 3 m, 12 m	2	Yes	[48, 58]
Tg4-42	Thy1 (Aβ4–42)	–	–	–	3 m	–	Yes	[10]
TBA42	Thy1 (Aβ3Q-42)	–	–	–	3 m	–	Yes	[77]
3xTg	Thy1 (AβPP, Tau) PS1 knock-in	Swe	M146V	P301L	7, 18 m	6	Yes	[49]
APP/PS1KI	Thy1 (AβPP) PS1 knock-in	Swe, Lon	M233, L235P	–	2, 6 m	2	Yes	[12]

Swe Swedish, *Flo* Florida, *Lon* London, *m* age in months

Table 3 Antibodies used in the study

Antibody	Source	Isotype	Aβ Epitope	Conformation	Clinical trial	References
Bapineuzumab	Humanized	IgG1	Recognizes the 5 extreme N-terminal residues (1–5)	Fibrils/plaques	Phase 2 (discontinued)	[44]
Solanezumab	Humanized	IgG1	Central region of Aβ (13–28)	Monomers	Phase 3 (in process)	[68]
Crenezumab	Humanized	IgG4	Central region of Aβ (12–23)	Monomer, oligomer, fibrils	Phase 2 (in process)	[14]
NTX4	Murine	IgG2b	N-terminus of $Aβ_{4-X}$ and pyroglutamate $Aβ_{3-X}$	Oligomers	–	[4]

Bright field images of DAB-immunostained tissue were acquired using a BX-51 microscope equipped with a Camera (Olympus, Tokyo, Germany). The semiquantitative analysis of plaque pathology and congophilic amyloid angiopathy (CAA) in formalin-fixed and paraffin-embedded tissue was based on Aβ staining intensity according to the following scale: −: no staining; (+): barely perceptible staining; +: weak staining; ++: moderate staining; +++: intense staining. All slides were assessed by two observers with no significant inter-observer variability. Figures were created with GraphPad Prism V6 for Windows (GraphPad Software, San Diego, CA, USA).

Amyloid plaque load quantification of human brain samples

Plaque load was quantified in formalin-fixed and paraffin-embedded human brain samples diagnosed with sporadic AD. Brains were stained with Bapineuzumab$_{BS}$, Crenezumab$_{BS}$, Solanezumab$_{BS}$ and NT4X. Furthermore, the pan-Aβ 2431-1 [10] was used as a control. For each of the 35 brains, four paraffin-embedded sections were stained simultaneously with DAB as chromogen. The relative Aβ load was evaluated in the frontal cortex using an Olympus BX-51 microscope equipped with an Olympus DP-50 camera and the ImageJ software (NIH, USA). Representative pictures of 20× magnification were systematically captured. Using ImageJ, the pictures were binarized to 8-bit black and white pictures and a fixed intensity threshold was applied defining the DAB staining. Measurements were performed for a percentage area covered by DAB staining. Statistics were calculated using GraphPad Prism V6 (GraphPad Software, San Diego, CA, USA) for Windows.

Results

The present report provides, to our best knowledge, the first comparative immunohistochemical analysis of Bapineuzumab$_{BS}$, Crenezumab$_{BS}$ and Solanezumab$_{BS}$ in human tissue and transgenic AD mouse models. The staining patterns of these humanized antibodies were also compared to the Aβ$_{4-x}$-specific antibody NT4X.

Bapineuzumab, Crenezumab, Solanezumab and NT4X demonstrated abundant vascular binding activity in sporadic Alzheimer's disease cases

To compare the staining patterns of Bapineuzumab$_{BS}$, Crenezumab$_{BS}$, Solanezumab$_{BS}$ and NT4X cortical tissue sections were analyzed in 35 sporadic AD cases and five non-demented control cases.

Cerebral amyloid angiopathy (CAA) staining of blood vessel walls was seen with Bapineuzumab$_{BS}$, Crenezumab$_{BS}$, Solanezumab$_{BS}$ and NT4X. All antibodies showed a comparable CAA staining pattern in parallel sections (Figs. 1, 3a–d). Vascular staining could be detected to variable degrees, using immunohistochemistry with Bapineuzumab$_{BS}$, Crenezumab$_{BS}$ and Solanezumab$_{BS}$, in all of the sporadic AD cases as well as in some non-demented control case we investigated. With NT4X vascular immunoreactivity was observed in 32 of 35 SAD cases, but in none of the control cases.

Bapineuzumab, Crenezumab and Solanezumab demonstrated abundant plaque binding activity in sporadic Alzheimer's disease cases

Bapineuzumab$_{BS}$ and Solanezumab$_{BS}$ showed a highly comparable staining profile and displayed abundant extracellular immunoreactivity in all patients using formalin-fixed and paraffin-embedded tissue (Figs. 2, 3e, g, i, k). Crenezumab$_{BS}$ showed a lower Aβ staining intensity in most cases than Bapineuzumab$_{BS}$ and Solanezumab$_{BS}$, although considerable plaque pathology was present in all of the sporadic AD cases (Figs. 2, 3f, j). This is consistent with the reported lower binding affinity of Crenezumab (nM) for Aβ than the homologous antibody Solanezumab (pM).

In contrast to Bapineuzumab$_{BS}$, Crenezumab$_{BS}$ and Solanezumab$_{BS}$, NT4X recognized only a minor portion of plaques in the brain tissue of AD patients. NT4X barely recognized plaques and in 68 % of the sporadic cases the antibody did not detect plaques at all (Figs. 2, 3e, l). Amyloid plaque load quantification of human brain samples confirmed these results (Fig. 4).

In addition abundant plaque pathology was also visualized with biotinylated Bapineuzumab$_{BS}$ (Fig. 5a, e), biotinylated Crenezumab$_{BS}$ (Fig. 5b, f), and biotinylated Solanezumab$_{BS}$ (Fig. 5c, g) in fresh frozen tissue. We biotinylated the humanized antibodies to avoid any possible compromise by the secondary antibody.

Comparative immunohistochemical analysis in different mouse models of AD

The mouse lines Tg4-42, TBA42, APP/PS1KI, 5XFAD and 3xTg (Table 2) were analyzed with Bapineuzumab$_{BS}$, Crenezumab$_{BS}$ and Solanezumab$_{BS}$ as well as with NT4X. These five transgenic mouse lines express different Aβ forms or combinations of human mutant APP, PS1 and Tau. It is well known that all of these mouse models develop plaques and/or intraneuronal Aβ at different stages during their life.

a

Case	Age	Gender	BS	Bapineuzumab	Crenezumab	Solanezumab	NT4X
Sporadic AD cases							
AD-1	79	f	IV	+	+	++	+
AD-2	81	f	IV	++	++	++	++
AD-3	84	f	IV	+	+	+++	+
AD-4	86	m	IV	+++	+++	+++	+++
AD-5	86	f	IV	+	+	+	++
AD-6	88	f	IV	+	+	+	+
AD-7	88	f	IV	+	+	+	+
AD-8	88	f	IV	+	+	+	+
AD-9	91	f	IV	+++	+++	+++	++
AD-10	91	m	IV	+++	+++	++	+
AD-11	92	f	IV	+	+	+	(+)
AD-12	93	m	IV	+++	+++	+++	+++
AD-13	94	f	IV	+++	+++	+++	+++
AD-14	68	f	V	+	+	+	(+)
AD-15	69	f	V	(+)	(+)	+	-
AD-16	78	f	V	++	++	+++	+
AD-17	78	f	V	+	+	+	+
AD-18	78	f	V	+	++	++	(+)
AD-19	79	f	V	++	+++	+++	++
AD-20	82	f	V	++	++	+++	++
AD-21	83	f	V	++	+	+++	+
AD-22	84	f	V	+++	+++	+++	+
AD-23	84	f	V	++	+	+	+
AD-24	84	f	V	++	++	++	(+)
AD-25	85	f	V	+++	+++	+++	+++
AD-26	85	f	V	+	+	+	+
AD-27	87	f	V	(+)	(+)	++	+
AD-28	88	f	V	+	++	++	++
AD-29	90	f	V	+	+	++	+
AD-30	58	f	VI	+	(+)	+	+
AD-31	62	f	VI	+	+	+	-
AD-32	65	f	VI	++	++	++	(+)
AD-33	65	f	VI	(+)	(+)	+	-
AD-34	70	f	VI	++	+++	+++	++
AD-35	86	f	VI	++	++	++	+
Non-demented control cases							
Control-1	91	m	I	-	-	-	-
Control-2	90	f	I	++	++	+	++
Control-3	84	m	I	-	-	-	-
Control-4	70	m	0	-	-	(+)	-
Control-5	70	m	0	++	++	++	+

b

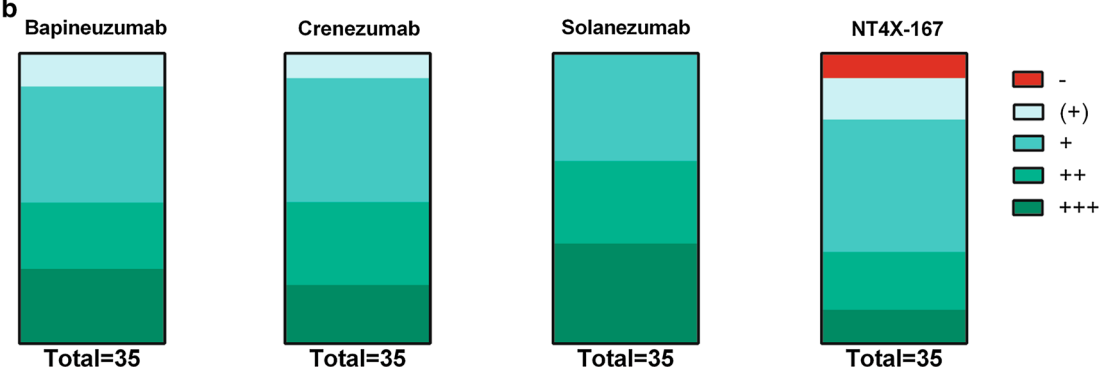

Fig. 1 Semiquantitative analysis of congophilic amyloid angiopathy (CAA) in sporadic and non-demented control cases. **a** List of demographic data and staining profiles of $Bapineuzumab_{BS}$, $Crenezumab_{BS}$, $Solanezumab_{BS}$ and NT4X in sporadic AD patients and non-demented control cases. **b** Summary of CAA staining profiles of $Bapineuzumab_{BS}$, $Crenezumab_{BS}$, $Solanezumab_{BS}$ and NT4X in SAD cases. Aβ staining intensity:− no staining; (+) barely perceptible staining; + weak staining; ++ moderate staining; +++ intense staining. *BS* Braak staging

a

Case	Age	Gender	BS	Bapineuzumab	Crenezumab	Solanezumab	NT4X
Sporadic AD cases							
AD-1	79	f	IV	++	+	+++	+
AD-2	81	f	IV	+++	++	+++	(+)
AD-3	84	f	IV	++	+	++	-
AD-4	86	m	IV	+	(+)	+	-
AD-5	86	f	IV	++	+	++	-
AD-6	88	f	IV	+++	(+)	++	-
AD-7	88	f	IV	++	+	+	-
AD-8	88	f	IV	+	+	+	-
AD-9	91	f	IV	++	+	+	-
AD-10	91	m	IV	++	+	++	(+)
AD-11	92	f	IV	+++	+	++	-
AD-12	93	m	IV	+	+	++	-
AD-13	94	f	IV	+++	+	+++	-
AD-14	68	f	V	+	+	+	-
AD-15	69	f	V	+	+	++	-
AD-16	78	f	V	++	++	+++	-
AD-17	78	f	V	+++	++	+++	(+)
AD-18	78	f	V	+++	+	+++	(+)
AD-19	79	f	V	++	++	+++	-
AD-20	82	f	V	++	+	++	-
AD-21	83	f	V	++	++	+++	(+)
AD-22	84	f	V	+++	++	+++	(+)
AD-23	84	f	V	++	++	++	-
AD-24	84	f	V	+++	++	+++	+
AD-25	85	f	V	+++	++	+++	-
AD-26	85	f	V	+++	++	+++	+
AD-27	87	f	V	+++	++	+++	-
AD-28	88	f	V	++	+	++	-
AD-29	90	f	V	++	+	++	-
AD-30	58	f	VI	+	+	+	-
AD-31	62	f	VI	++	+	++	-
AD-32	65	f	VI	++	+	++	(+)
AD-33	65	f	VI	+	+	+	-
AD-34	70	f	VI	++	+	+	-
AD-35	86	f	VI	+++	++	++	+
Non-demented control cases							
Control-1	91	m	I	-	-	-	-
Control-2	90	f	I	(+)	(+)	+	-
Control-3	84	m	I	-	-	-	-
Control-4	70	m	0	-	-	-	-
Control-5	70	m	0	++	++	++	-

b

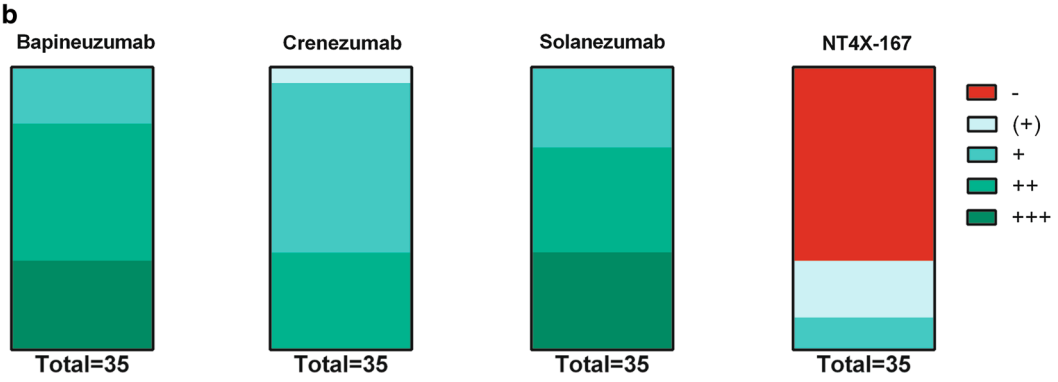

Fig. 2 Semiquantitative analysis of plaque pathology in sporadic and non-demented control cases. **a** List of demographic data and staining profiles of $Bapineuzumab_{BS}$, $Crenezumab_{BS}$, $Solanezumab_{BS}$ and NT4X in sporadic AD patients and non-demented control cases. **b** Summary of plaque staining profiles of $Bapineuzumab_{BS}$, $Crenezumab_{BS}$, $Solanezumab_{BS}$ and NT4X in SAD cases. Aβ staining intensity: − no staining; + barely perceptible staining; + weak staining; ++ moderate staining; +++ intense staining. *BS* Braak staging

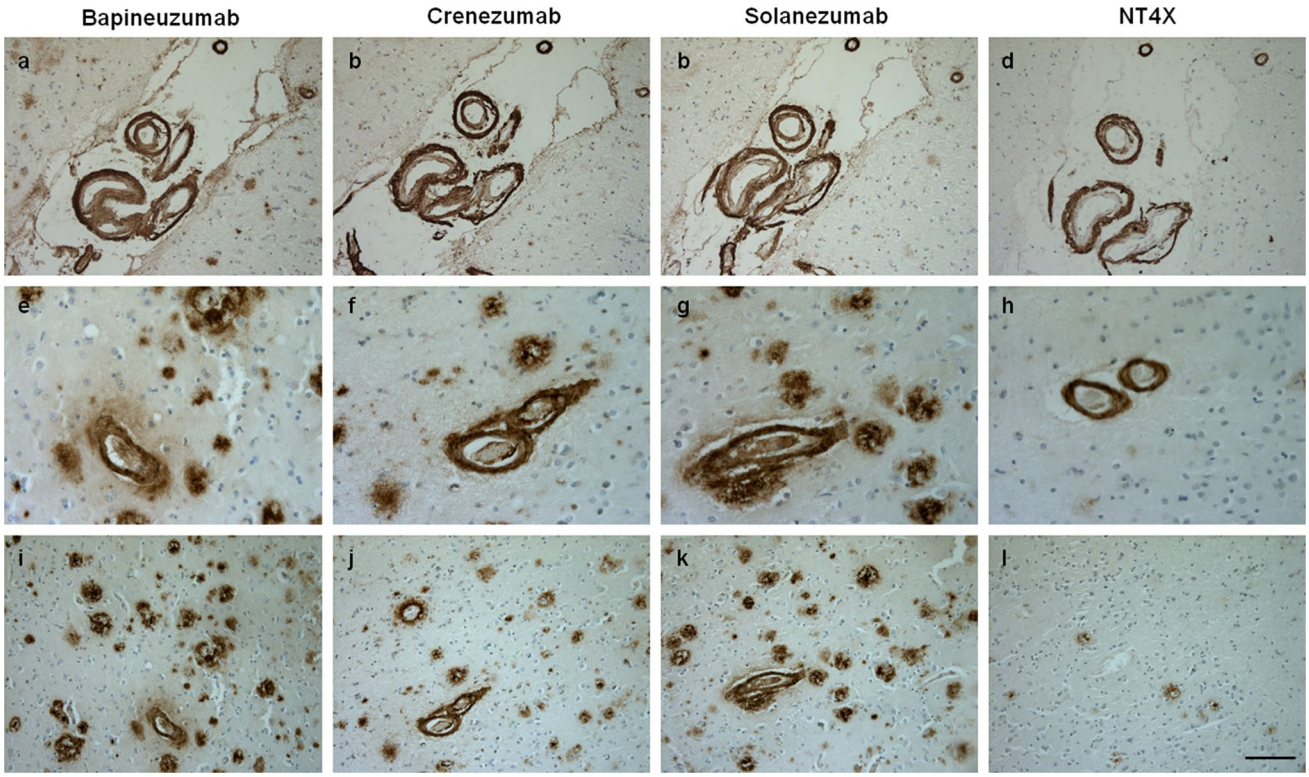

Fig. 3 Immunohistochemical staining pattern of sporadic AD brains. Cerebral amyloid angiopathy (CAA) visualized with Bapineuzumab$_{BS}$ (**a**), Crenezumab$_{BS}$ (**b**), Solanezumab$_{BS}$ (**c**) and NT4X (**d**). All antibodies showed a comparable CAA staining pattern in cortical parallel sections (**a**–**d**). Abundant plaque pathology could be recognized with Bapineuzumab$_{BS}$ (**a**, **e**, **i**), Crenezumab$_{BS}$ (**b**, **f**, **j**), and Solanezumab$_{BS}$ (**c**, **g**, **k**). In contrast, NT4X showed a preferential binding to blood vessels presenting CAA and barely recognized plaques (**d**, **h**, **i**). *Scale bar* **a**–**d**, **i**–**l** 100 µm; **e**–**h** 50 µm

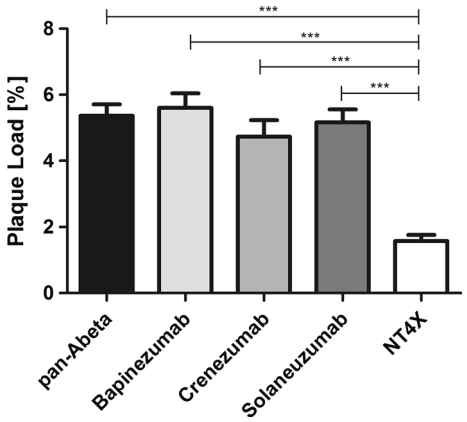

Fig. 4 Plaque load quantification in sporadic AD cases. Immunostaining with Bapineuzumab$_{BS}$, Crenezumab$_{BS}$, Solanezumab$_{BS}$ and the pan-Abeta antibody 2431-1 revealed no significant differences in the detection of plaques in sporadic AD cases. NT4X detected significantly less plaques in sporadic AD cases than Bapineuzumab$_{BS}$, Crenezumab$_{BS}$, Solanezumab$_{BS}$ and pan-Abeta. One-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test; $n = 35$; ***$p < 0.001$, data presented as mean ± SEM

Staining of *3xTg* mice expressing both mutant APP and mutant Tau on a PS1 knock-in background revealed intraneuronal Aβ in the cortex of 18-month-old 3xTg mice with Bapineuzumab$_{BS}$, Crenezumab$_{BS}$, Solanezumab$_{BS}$ and NT4X (Fig. 6a–d). Furthermore, abundant extracellular plaque staining was visible with all antibodies at the age of 18 months in the subiculum of the hippocampus (Fig. 6e–h). Intraneuronal Aβ and plaque staining was less abundant with Crenezumab$_{BS}$ and NT4X.

APP/PS1KI mice overexpress human APP with the Swedish and London mutations on a mutant PS1 knock-in background. Abundant plaque pathology was observed among others in the cortex and hippocampus at 6 months of age (Fig. 7e–l). Furthermore, all four antibodies demonstrated intraneuronal Aβ accumulation and extracellular plaques in 2-month-old mice (Fig. 7a–d). Staining in APP/PS1KI was most abundant with Bapineuzumab$_{BS}$ at both ages.

To compare the staining pattern of NT4X, Bapineuzumab$_{BS}$, Crenezumab$_{BS}$ and Solanezumab$_{BS}$ in the well-established *5XFAD* mouse model hemizygous and homozygous

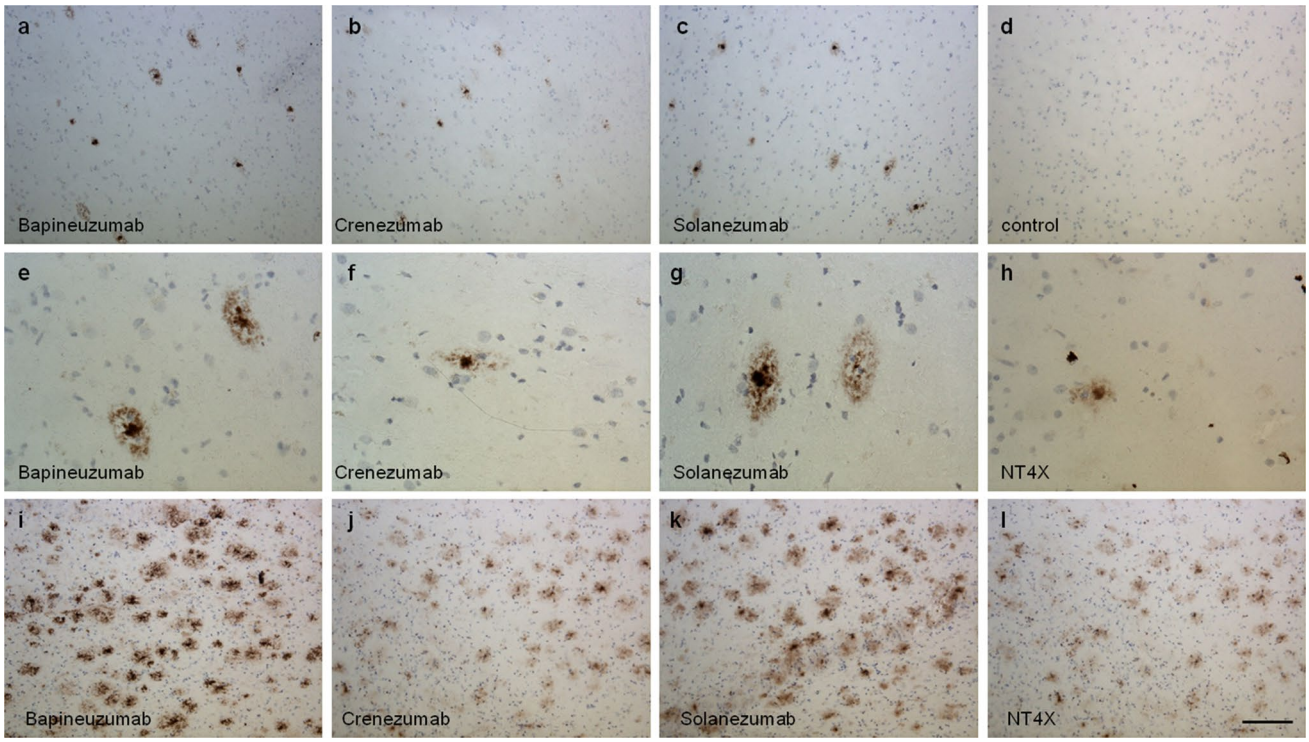

Fig. 5 Immunohistochemical staining pattern in fresh frozen tissue. Abundant plaque pathology visualized with biotinylated Bapineuzumab$_{BS}$ (**a**, **e**), biotinylated Crenezumab$_{BS}$ (**b**, **f**), and biotinylated Solanezumab$_{BS}$ (**c**, **g**) in cortical human tissue. Only weak plaque staining with NT4X in fresh frozen human tissue (**d**). Abundant plaque pathology could be recognized with biotinylated Bapineuzumab$_{BS}$ (**i**), biotinylated Crenezumab$_{BS}$ (**j**), biotinylated Solanezumab$_{BS}$ (**k**) and NT4X (**l**) in aged 5XFAD mice. No staining was observed under control conditions with ABC incubation alone (**d**) *Scale bar* **a**–**d**, **j**–**l** 100 μm; **e**–**h** 50 μm

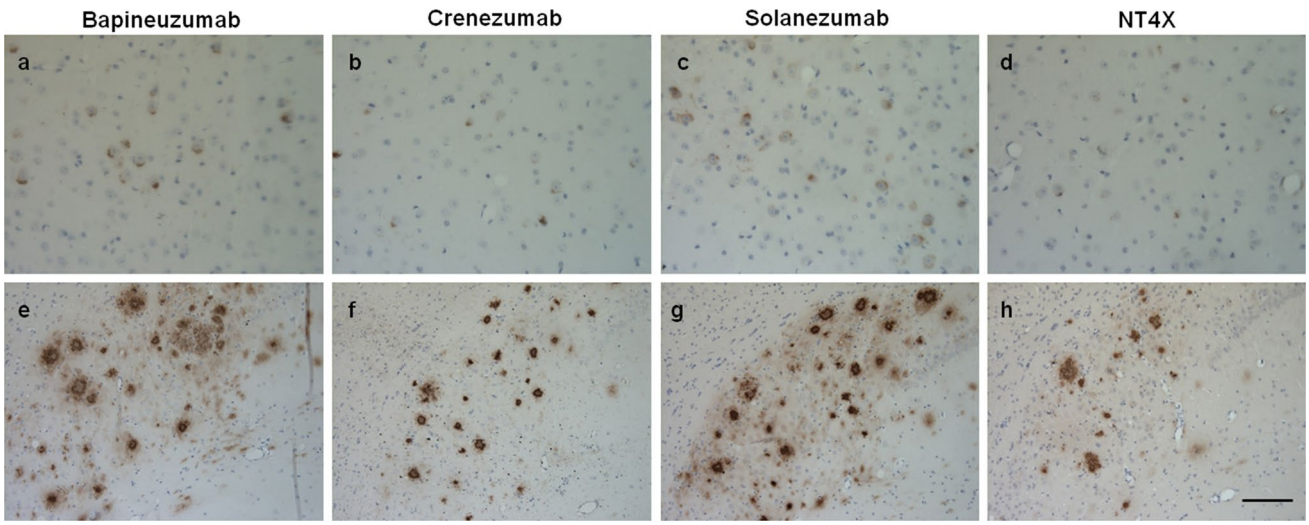

Fig. 6 Immunohistochemical staining of 3xTg transgenic mice. Intraneuronal Aβ could be detected with Bapineuzumab$_{BS}$ (**a**), Crenezumab$_{BS}$ (**b**), Solanezumab$_{BS}$ (**c**) and NT4X (**d**) in the cortex of 18-month-old 3xTg mice. Abundant extracellular plaque staining visible with all antibodies at the age of 18 months in 3xTg mice (**e**–**h**). Intraneuronal Aβ and plaque staining was less abundant with Crenezumab$_{BS}$ (**b** and **f**) and NT4X (**d** and **h**). *m* month. *Scale bar* **a**–**d** 50 μm; **e**–**h** 100 μm

mice were studied using cortical and hippocampal sections, as these are the brain areas known to develop abundant intraneuronal and extraneuronal Aβ pathology. Immunostaining with all four antibodies demonstrated strong intraneuronal Aβ accumulation in 6-week-old homozygous 5XFAD (Fig. 7a–d). As expected, intraneuronal Aβ accumulation

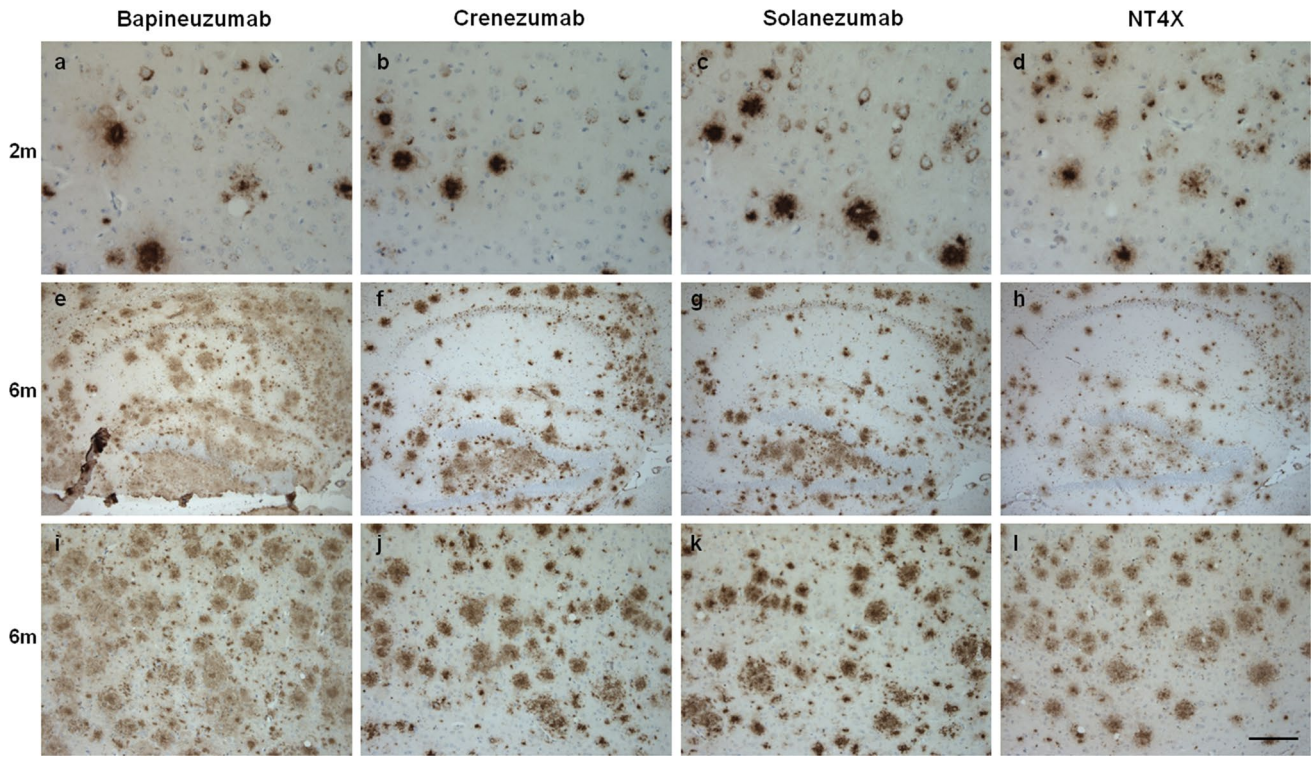

Fig. 7 Immunohistochemical staining of hippocampal and cortical sections of APP/PS1KI transgenic mice. Immunostaining with Bapineuzumab$_{BS}$ (**a**), Crenezumab$_{BS}$ (**b**), Solanezumab$_{BS}$ (**c**) and NT4X (**d**) demonstrating intraneuronal Aβ accumulation and extracellular plaques in 2-month-old APP/PS1KI mice. Abundant extracellular plaque staining in the hippocampus (**e**–**h**) and cortex (**i**–**l**) with all four antibodies at 6 months of age. Staining was most abundant with Bapineuzumab$_{BS}$. *m* months. *Scale bar* **a**–**d** 50 μm; **i**–**l** 100 μm; **e**–**h** 200 μm

was observed in young, but not in aged 5XFAD mice. 5XFAD mice, overexpressing APP with the Swedish, Florida and London mutations combined with PSEN1 carrying the M146L and L286V mutations, showed abundant extracellular plaque staining with Bapineuzumab$_{BS}$ in the hippocampus and cortex of 3-month-old hemizygous 5XFAD mice (Fig. 8e, i). Plaque staining in young 5XFAD mice was less abundant with Solanezumab$_{BS}$ (Fig. 8g, k). Only faint plaque staining was observed with Crenezumab$_{BS}$ and NT4X at 3 months of age (Fig. 8f, j, h, l). Furthermore, immunohistochemical detection of Aβ, using Bapineuzumab$_{BS}$, Crenezumab$_{BS}$, Solanezumab$_{BS}$ and NT4X, showed an abundant plaque-associated pathology in 12-month-old 5XFAD mice (Fig. 8m–p). Abundant plaque pathology could also be recognized with biotinylated Bapineuzumab$_{BS}$ (i), biotinylated Crenezumab$_{BS}$ (j), biotinylated Solanezumab$_{BS}$ (k) and NT4X (l) in aged 5XFAD mice in fresh frozen tissue.

In 3-month-old homozygous *Tg4-42* mice, expressing Aβ$_{4–42}$, strong intraneuronal immunoreactivity could be detected with Crenezumab$_{BS}$, Solanezumab$_{BS}$ and NT4X. The three antibodies detected intraneuronal Aβ in the CA1 region of the hippocampus (Fig. 9b–d), where staining was most intense with Solanezumab$_{BS}$. In contrast, no intraneuronal Aβ was observed with Bapineuzumab$_{BS}$ in Tg4-42 mice (Fig. 9a). The inability of Bapineuzumab$_{BS}$ to detect Aβ in *Tg4-42* mice is consistent with the antibodies affinity for the N-terminus of Abeta 1−x in a helical conformation stabilized by hydrogen bonds involving amino acids 1–3, which are absent in this model [44].

In *TBA42* mice that overexpress pyroglutamate Aβ$_{3–42}$, intraneuronal Aβ has previously been reported predominately in the CA1 of the hippocampus [77]. However, intraneuronal Aβ in hippocampal TBA42 brain sections could only been detected with Solanezumab$_{BS}$ (Fig. 9g). In contrast, Bapineuzumab$_{BS}$, Crenezumab$_{BS}$ and NT4X showed no staining in 3-month-old TBA42 mice. This is consistent with the epitope specificities of Bapineuzumab$_{BS}$ and NT4X. In the case of Crenezumab$_{BS}$, this is likely due simply to the significantly lower affinity of this antibody relative to the homologous antibody Solanezumab$_{BS}$.

Discussion

Currently, immunotherapy is considered to be among the most advanced and promising approaches to prevent or

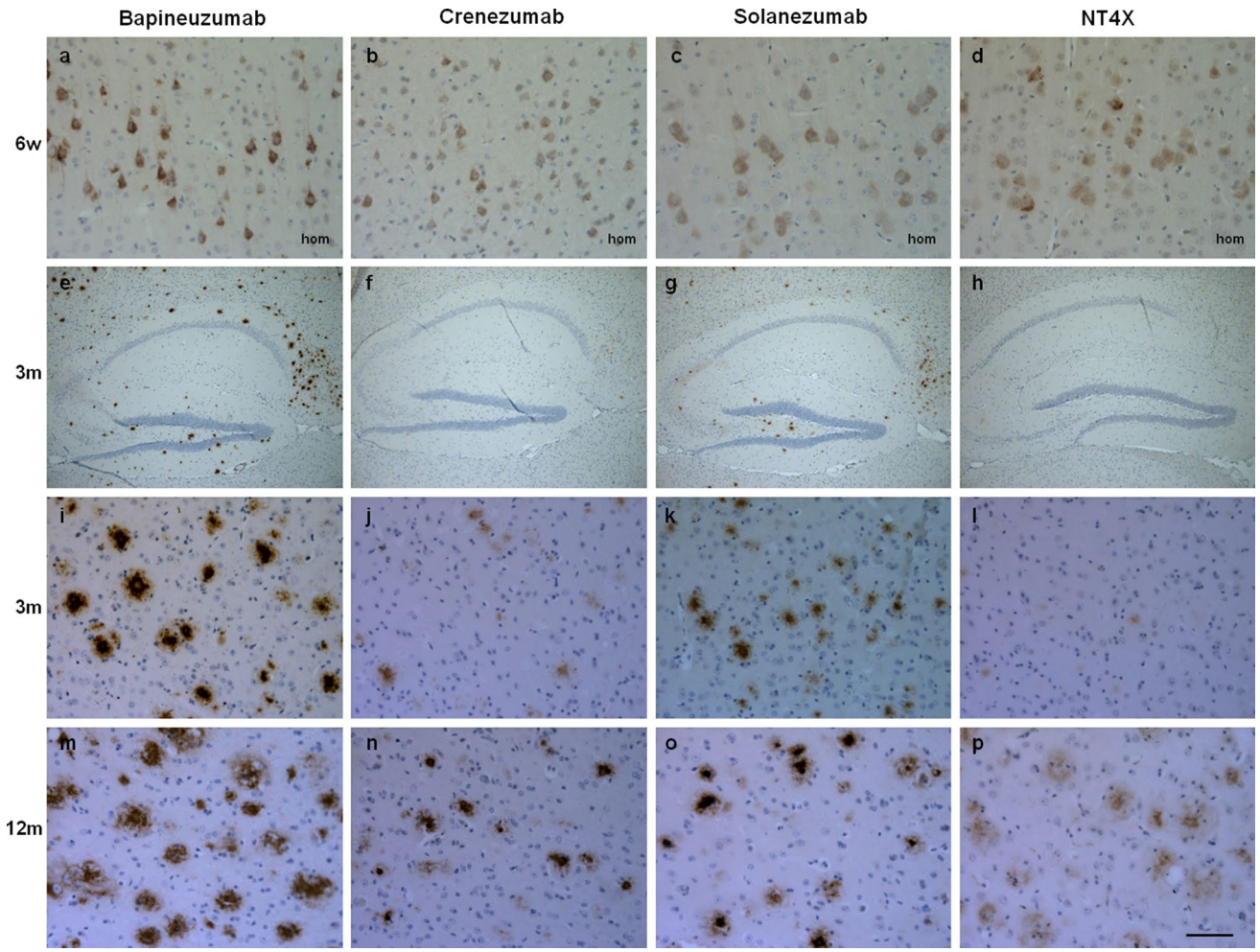

Fig. 8 Immunohistochemical staining of hippocampal and cortical sections of 5XFAD transgenic mice. Immunostaining with Bapineuzumab$_{BS}$ (**a**), Crenezumab$_{BS}$ (**b**), Solanezumab$_{BS}$ (**c**) and NT4X (**d**) demonstrating intraneuronal Aβ accumulation in 6-week-old homozygous 5XFAD. Abundant extracellular plaque staining with Bapineuzumab$_{BS}$ at the age 3 months in hemizygous 5XFAD (**e** and **i**). Plaque staining in 5XFAD mice was less abundant with Solanezumab$_{BS}$ (**g** and **k**). Only faint plaque staining was observed with Crenezumab$_{BS}$ (**f** and **j**) and NT4X (**h** and **l**) at 3 months of age. Abundant extracellular plaque staining with Bapineuzumab$_{BS}$ (**m**), Crenezumab$_{BS}$ (**n**), Solanezumab$_{BS}$ (**o**) and NT4X (**p**) at the age of 12 months. *m* months, *w* weeks, *hom* homozygous. *Scale bar* **a**–**d**; **i**–**p** 50 μm; **e**–**h** 200 μm

treat AD. The first evidence that antibodies are potential therapeutic agents for AD was reported in 1996. Solomon et al. [67] demonstrated that antibodies against the N-terminal region of Aβ prevented the formation of Aβ fibrils in vitro, disaggregated pre-formed fibrils and evoked neuroprotection against Aβ toxicity [66]. In 1999, Schenk and colleagues demonstrated in vivo that active immunization with pre-aggregated synthetic Aβ$_{1–42}$ could prevent the onset of plaque formation in young mice and decelerate the progression of plaque formation in PDAPP mice [61]. Several other studies replicated these results in different mouse models suggesting that they could be translated to the clinic [15, 31, 72]. The first anti-Aβ clinical trial AN1792 used full-length Aβ$_{1–42}$ in an active immunization trial, but was terminated in phase II as several treated patients developed a severe T cell-mediated immune response to the antigen giving rise to meningoencephalitis [21, 50].

Consequently, passive immunization was considered as a safer and more controllable alternative to active immunization. It was shown that peripheral administration of a monoclonal antibody against amyloid β-peptide (3D6 murine parent antibody of Bapineuzumab) was sufficient to reduce the amyloid load in PDAPP mice [5]. Further studies also demonstrated that the passive immunization approach significantly reduced CNS Aβ and restored memory deficits in the Morris Water Maze and object recognition in APP mice [11, 18, 33]. Due to the promising results in mice and the lack of possible Th-1 mediated autoimmunity, passive immunization was promptly moved to the clinic [76]. So far several different antibodies have been tested in

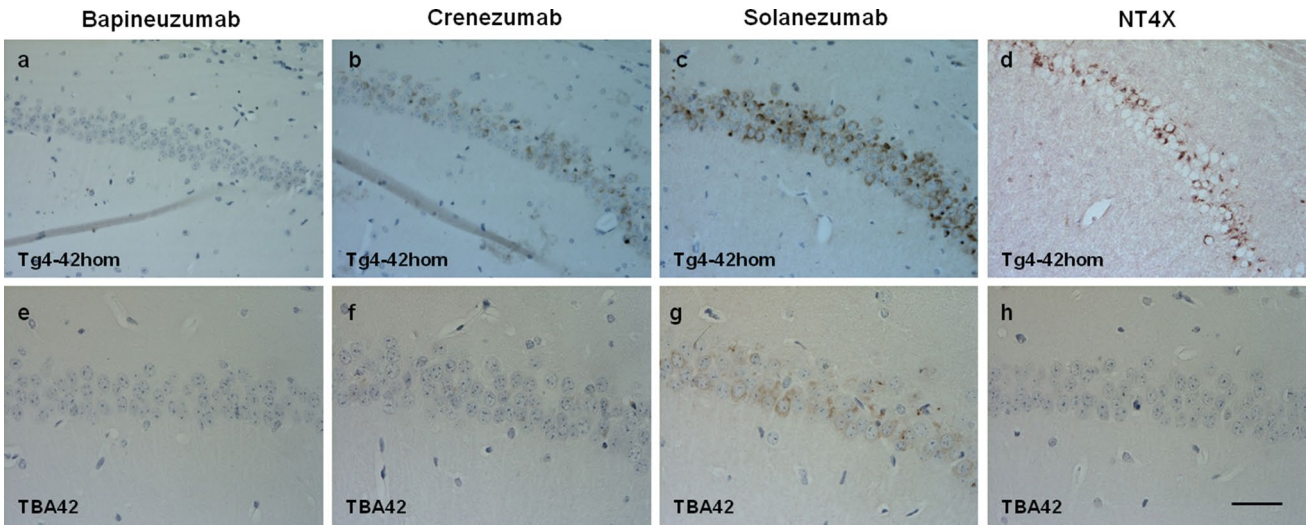

Fig. 9 Immunohistochemical staining of hippocampal sections of Tg4-42 and TBA42 transgenic mice. Positive intraneuronal immunoreactivity could be detected with Crenezumab$_{BS}$ (**b**), Solanezumab$_{BS}$ (**c**) and NT4X (**d**) in homozygous Tg4-42 mice. All three antibodies detected intraneuronal Aβ in the hippocampus of 3-month-old mice. No intraneuronal Aβ was observed with Bapineuzumab$_{BS}$ (**a**). Intraneuronal Aβ in hippocampal TBA42 brain sections was only detected with Solanezumab$_{BS}$ (**g**). In contrast, Bapineuzumab$_{BS}$ (**e**), Crenezumab$_{BS}$ (**f**) and NT4X (**h**) showed no parenchymal staining in 3-month-old TBA42 mice. *hom* homozygous. *Scale bar* **a**–**d** 50 μm; **e**–**h** 33 μm

clinical trials including Bapineuzumab, Crenezumab and Solanezumab.

Bapineuzumab (Pfizer and Johnson & Johnson) is a humanized IgG1 antibody directed against the N-terminus of Aβ recognizing the Aβ$_{1–5}$ region. It is the first antibody tested in a passive immunization trial for AD [51, 68]. Two large clinical trials with mild to moderate AD patients revealed no significant benefits, leading to the termination of all phase III studies in 2012 [38, 51]. Despite the lack of cognitive improvements Bapineuzumab was able to stabilize the plaque burden and lower phosphorylated-tau levels in cerebrospinal fluid [44]. Bapineuzumab and its murine form 3D6 are reported to bind with a high affinity to various Aβ forms without cross-reacting with APP [5, 6, 36, 61, 78]. Bapineuzumab binds to the N-terminus of Aβ in a helical conformation. The N-terminus created by BACE cleavage is buried in the antibody surface, and the helical conformation might provide a possible mechanism for the antibody to exhibit differential binding to monomeric and aggregated pools of Aβ depending on the exhibition of this epitope [20, 44]. Well in line with previous findings we could show that Bapineuzumab binds strongly to plaques in transgenic APP mice as well as vascular amyloid and Aβ plaques in human formalin-fixed, paraffin-embedded tissue and fresh frozen tissue.

The antibodies Crenezumab (Genentech) and Solanezumab (Eli Lilly) are reported to be highly homologous in their antigen binding region [14, 71] and are currently in clinical phase II or III trials [1].

Crenezumab is derived from a mouse antibody binding to Aβ$_{12–23}$ and is reported to bind Aβ monomers, oligomers and fibrils with equal high affinity [2]. In contrast to Bapineuzumab and Solanezumab, the humanized antibody is designed on an IgG4 backbone to reduce the risk of microglial-mediated pro-inflammatory effects, including vasogenic edema [2]. A phase I clinical trial proved safety of Crenezumab and the antibody is currently in phase II trial [1].

Here, we demonstrated a high binding affinity of Crenezumab$_{BS}$ in formalin-fixed, paraffin-embedded tissue and fresh frozen tissue to vascular amyloid and plaques in sporadic AD cases, however, the plaque staining intensity with Crenezumab$_{BS}$ was in most cases lower than with Bapineuzumab$_{BS}$ and Solanezumab$_{BS}$. Furthermore, abundant plaque pathology was detected in 5XFAD, 3xTg and APP/PS1KI mice.

Similar to Crenezumab, Solanezumab is targeted to an internal epitope of Aβ (13–28) [68]. The antibody is an IgG1 monoclonal antibody derived from the murine antibody 266 that preferentially binds soluble Aβ [16]. Early clinical trials with Solanezumab showed increased levels of plasma and CSF Aβ and showed little cognitive improvements in patients with moderate AD [19]. Currently, a large phase III trial with Solanezumab is ongoing.

Solanezumab is reported to recognize various forms of N-truncated Aβ [30]. Our findings are in line with these reports, as Solanezumab$_{BS}$ detected intraneuronal pyroglutamated Aβ$_{3–x}$ and Aβ$_{4–x}$ in TBA42 and Tg4-42 mice

in formalin-fixed, paraffin-embedded tissue, respectively. Importantly, these mice do only express N-truncated Aβ variants pyroglutamated $Aβ_{3-x}$ and $Aβ_{4-x}$ and not APP.

Surprisingly, $Solanezumab_{BS}$ detected abundant plaque pathology in all of the 35 analyzed sporadic AD cases and in two of the control cases in formalin-fixed and paraffin-embedded tissue as well as in fresh frozen tissue. Furthermore, the antibody also detected plaques in 5XFAD, APP/PS1KI and 3xTg mice. In contrast to these results, it has previously been reported that the antibody inhibits fibril formation [37] by synthetic Aβ and only detects soluble monomeric Aβ [46]. Solanezumab has nearly the same sequence of amino acids interacting with Aβ as Crenezumab suggesting that the two antibodies bind Aβ in the same manner [14]. Interestingly, while Solanezumab is not reported to bind aggregated forms of Aβ its murine parent was reported to do so [65]. Seubert et al. showed that 'antibody 266 (IgG1 isotype) recognizes a central epitope of Aβ residues 16–24 and much less effectively binds amyloid plaques, as compared to 3D6'.

Next to Aβ starting with aspartate as the first amino acid, a variety of modified and N-truncated Aβ variants has been characterized [34]. N-truncated Aβ can be found plaque associated as well as intraneuronal in AD mouse models [25] and it has been demonstrated that N-terminal deletions enhance aggregation of β-amyloid into neurotoxic, β-sheet fibrils. Such peptides may initiate and/or nucleate the pathological deposition of Aβ into plaques [52]. Furthermore, Sergeant et al. [64] demonstrated that amino-truncated Aβ species represent a large portion of all Aβ species in fully developed AD as well as at the earliest stage of AD pathology and are only detected during disease progression [7]. It was concluded that therapy specifically targeting these pathological amino-truncated species of $Aβ_{x-42}$ might be therapeutically advantageous.

Several pre-clinical studies already showed beneficial effects of passive immunization against pyroglutamate $Aβ_{3-x}$. Pyroglutamated $Aβ_{3-x}$ mAbs reduced plaque depositions while limiting the side effects of vaccination. For example, passive immunization of 5XFAD mice with the 9D5 antibody that detects low molecular weight pyroglutamate Aβ oligomers reduced Aβ plaque load and $Aβ_{pE3-x}$ levels in mice [75]. Another anti-pyroglutamated $Aβ_{3-x}$ antibody (mAb07/1) significantly reduced plaque burden after passive immunization of APPswe/PS1dE9 transgenic mice without increasing vascular amyloid or microhemorrhage [23]. Similarly, De Mattos and colleagues reported that passive immunization of PDAPP mice reduced pre-existing plaques without inducing microhemorrhage in a dose-dependent manner [17].

Here, we tested the binding affinity of $Bapineuzumab_{BS}$, $Crenezumab_{BS}$ and $Solanezumab_{BS}$ to pyroglutamated $Aβ_{3-x}$ in TBA42 mice in formalin-fixed and paraffin-embedded tissue. This mouse model expresses only $Aβ_{3Q-42}$ starting with an N-terminal glutamine (Q) residue at position three of Aβ as glutamine is a better substrate for both the spontaneous and enzymatically catalyzed conversion of $Aβ_{3-42}$ into $Aβ_{pE3-42}$ [43, 77]. Therefore, TBA42 mice do not express human APP or other Aβ variants. Only $Solanezumab_{BS}$ was able to detect intraneuronal Aβ in the CA1 of the hippocampus in these mice, while the highly homologous $Crenezumab_{BS}$ did not detect Aβ in this mouse model. This is likely not a consequence of specificity, but of binding affinity, consistent with the three orders of magnitude lower binding affinity of $Crenezumab_{BS}$ for Aβ relative to $Solanezumab_{BS}$. As expected, $Bapineuzumab_{BS}$ showed no parenchymal staining in TBA42 mice as it has been previously shown that Bapineuzumab is not able to recognize N-terminally modified or truncated Aβ species [44]. The crystal structure of a Bapineuzumab Fab-Aβ peptide complex revealed that it captured Aβ in a monomeric helical conformation at the N-terminus terminus and in doing so buried the N-terminus of the peptide [44].

Next to pyroglutamate $Aβ_{3-42}$ $Aβ_{4-42}$ could be another potential therapeutic target for AD. This peptide aggregates irreversibly and rapidly into soluble toxic oligomers and only slowly reacts further into inert amorphous fibrils [10]. $Aβ_{4-42}$ is very abundant in AD and was discovered as early as 1985 [40]. However, $Aβ_{4-x}$ has not received much attention as a potential therapeutic target. McLaurin et al. [41] have performed an active immunization approach in TgCRND8 transgenic mice using protofibrillar $Aβ_{1-42}$ peptides. The mice developed robust titers against Aβ and the sera isolated from these mice stained mature, but not diffuse plaques in TgCRND8 mice. The therapeutically active antibodies were subsequently isolated and characterized. Interestingly, although protofibrillar $Aβ_{1-42}$ was used as vaccine, beneficial effects in mice arose from antibodies selectively directed against residues 4–10 of Aβ. These antibodies inhibited both Aβ fibrillogenesis and cytotoxicity without eliciting an inflammatory reaction. It seems likely that pre-clinical immunization trials against $Aβ_{4-42}$ are only a matter of time.

$Crenezumab_{BS}$ and $Solanezumab_{BS}$ were able to detect intraneuronal Aβ in the hippocampus of Tg4-42 mice, expressing $Aβ_{4-42}$. Again $Bapineuzumab_{BS}$ did not recognize the truncated Aβ species in formalin-fixed and paraffin-embedded tissue. As expected, our recently generated the $Aβ_{4-x}$-specific antibody NT4X showed positive intraneuronal immunoreactivity in Tg4-42 mice expressing only $Aβ_{4-42}$ and not human APP or any other Aβ variant.

NT4X is able to differentiate between full-length Aβ and the two major N-truncated variants, $Aβ_{4-x}$ and $Aβ_{pE3-x}$. NT4X significantly rescued $Aβ_{4-42}$ toxicity in vitro, while no beneficial effect was observed against $Aβ_{1-42}$ or $Aβ_{pE3-42}$ toxicity [4]. Here, we extended our previous findings and

demonstrated that the antibody detects plaques in APP/PS1KI, 5XFAD and 3xTg mice, but barely reacted with plaques in the brain of sporadic AD patients in formalin-fixed, paraffin-embedded tissue or fresh frozen tissue. These data are corroborated by a previous work which analyzed Aβ pathology using chemical and morphological approaches comparing the plaques of APP23 transgenic mice and human AD brain [35]. Chemical analyses revealed that the amyloid plaque cores in APP23 transgenic mice were completely soluble in buffers containing SDS despite an obvious overall structural resemblance to AD pathology. Human AD plaque cores were highly resistant to chemical and physical disruption accounting for the extreme stability of AD plaque cores. An explanation for the differences in solubility between human AD and the APP23 mouse plaques may be due to the lack of post-translational modifications including N-terminal degradation, isomerization, racemization, pyroglutamyl formation and oxidation of transgenic mouse Aβ [35]. NT4X preferably stained Aβ in blood vessels in humans, in which $Aβ_{x\text{-}40}$ is a major component.

The weak binding of NT4X to plaques in human tissue could be construed as a potential therapeutic advantage. The strategy of using antibodies that target mainly plaques is increasingly being regarded as a potential risk factor as plaques may serve as reservoirs of toxic Aβ peptides [8, 28]. The failure of Bapineuzumab may at least be partly explained by that hypothesis.

While the insoluble fibrillar aggregates of amyloid-β are the main neuropathological hallmark of AD, the plaque load correlates poorly with brain dysfunction and cognitive impairment in AD patients [39, 54] or in AD transgenic mouse models [45, 62]. In contrast, recent studies indicate that soluble Aβ levels, including soluble oligomers, correlate much better with key features of AD [42, 47, 63]. Furthermore, growing evidence suggests that intraneuronal Aβ accumulation contributes to the pathological events in AD. Intraneuronal Aβ accumulation has also been detected in a variety of AD mouse models including Tg2576, 3xTg, APP/PS1KI and 5XFAD [48, 49, 70]. Early and transient intraneuronal accumulation of Aβ correlated with subsequent neuron loss also in diverse APP/Aβ transgenic mouse models and brain regions [3, 10, 13, 43, 74]. Here, we demonstrated that the three humanized antibodies $Bapineuzumab_{BS}$, $Crenezumab_{BS}$ and $Solanezumab_{BS}$ were able to detect intraneuronal Aβ in young 5XFAD and APP/PS1KI as well as in aged 3xTg mice. Cross-reactivity with APP cannot be ruled out entirely in these models though. NT4X, specific to the N-terminus of $Aβ_{4-x}$ and $Aβ_{pE3-x}$, also showing intraneuronal staining in these mouse lines underlines the abundance of $Aβ_{4-x}$ and $Aβ_{pE3-x}$ in these models of AD. Interestingly, Grundke-Iqbal et al. [27] reported already 25 years ago that intracellular Aβ may precede Aβ plaque formation in AD patients. It should be noted, however, that the existence of intraneuronal Aβ especially in human AD brain sections is disputed in the field [26]. The major information using transgenic mice in the present study came from mice expressing N-truncated $Aβ_{4–42}$ and pyroglutamate $Aβ_{3–42}$ as we demonstrated the difference in target engagement among the four antibodies studied.

It has to be noted that the human antibodies used in the current study are 'bio-similar' versions based on the available patent literature rather than the original antibodies [14, 44, 71]. Furthermore, it has to be noted that while immunohistochemistry is one of the pillars of diagnostic pathology and an important research tool in pathology and research, the results obtained with immunohistochemistry may not accurately reflect in vivo conditions. Nevertheless, it is an important part of the characterization and comparison of antibodies.

In summary, the present report describes the immunohistochemistry profile of $Bapineuzumab_{BS}$, $Crenezumab_{BS}$ and $Solanezumab_{BS}$ in human and mouse tissue. All three antibodies showed a very similar staining profile in formalin-fixed and paraffin-embedded tissue and fresh frozen tissue and were able to detect plaques and CAA in humans in addition to plaques and intraneuronal Aβ in mice. $Solanezumab_{BS}$ was the only antibody able to detect not only $Aβ_{4–42}$ but also pyroglutamate $Aβ_{3–42}$. In contrast, NT4X showed a low binding affinity to plaques in human tissue while recognizing CAA as well as intraneuronal Aβ and plaques in mice.

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Compliance with ethical standards

Conflict of interest A patent application for the antibody NT4X was filed by the University Medicine of Goettingen and Thomas A. Bayer.

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