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Immune activation in Aβ-related angiitis (ABRA) and a cell model of E280A presenilin-mutated Familial Alzheimer's Disease

Dissertation

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Immune Activation in Amyloid-β-Related Angiitis Correlates with Decreased Parenchymal Amyloid-β Plaque Load

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Abstract

Background: Primary angiitis of the central nervous system (PACNS) is a rare but serious condition. A fraction of patients suffering from PACNS concurrently exhibit pronounced cerebral amyloid angiopathy (CAA) which is characterized by deposits of amyloid-β (Aβ) in and around the walls of small and medium-sized arteries of the brain. PACNS with CAA has been identified as a distinct disease entity, termed Aβ-related angiitis (ABRA). Evidence points to an immune reaction to vessel wall Aβ as the trigger of vasculitis. ***Objective:*** To investigate whether the inflammatory response to Aβ has (1) any effect on the status of immune activation in the brain parenchyma and (2) leads to clearance of Aβ from brain parenchyma. ***Methods:*** We studied immune activation and Aβ load by quantitative immunohistochemical analysis in brain parenchyma adjacent to affected vessels in 11 ABRA patients and 10 matched CAA controls. ***Results:*** ABRA patients showed significantly increased immune activation and decreased Aβ loads in the brain parenchyma adjacent to affected vessels. ***Conclusion:*** Our results are in line with the hypothesis of ABRA being the result of an excessive immune response to Aβ and show that this can lead to enhanced clearance of Aβ from the brain parenchyma by immune-mediated mechanisms.

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Introduction

Primary angiitis of the central nervous system (PACNS) is defined by focal, inflammatory vascular lesions confined to the brain and spinal cord without systemic involvement. PACNS is rare, with an estimated incidence of 2–3 cases per million per year, a median age of 47 years, a progressive clinical course with frequent recurrences and high lethality if not adequately treated [1].

Cerebral amyloid angiopathy (CAA) is a common pathological finding in the elderly, characterized by deposition of amyloid-β (Aβ) within the cortical and meningeal arteries [2]. Its prevalence increases from 2–3% at the age of 70 to 15–30% at the age of 90 [2]. In Alzheimer's

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disease (AD), the prevalence of CAA is very high, possibly reaching 90% [2, 3]. Almost a third of intracranial lobar hemorrhages in the elderly are attributed to CAA-induced damage to vessel walls [2].

In certain instances, patients present with coincident Aβ deposition in vessel walls and angiitis [4, 5]. A variety of names such as granulomatous angiitis with CAA, inflammatory CAA or Aβ-related angiitis (ABRA) have been used to describe this clinicopathological entity [4–6]. Clinical and neuropathological investigations on ABRA have provided valuable insights into the pathogenesis of this disease [7–9]. Regarding its pathogenesis, there are at least three distinct possibilities: (1) Aβ deposition in vessel walls and angiitis concur by chance as stochastic events, (2) angiitis favors vessel wall deposition of Aβ [10] or (3) Aβ deposition in vessel walls leads to angiitis [5].

In a large successive case series of biopsy-proven PACNS, the co-occurrence of vascular Aβ and angiitis was recently studied [11–12]. Histological evidence of ABRA was detected in approximately 30% of PACNS with an average age of 46 years. Since the prevalence of CAA in this age group is 2–3% [2], chance association is highly unlikely. Thus, it seems that vascular Aβ deposits and angiitis are pathophysiologically interconnected. None of the studies on ABRA has provided convincing evidence that angiitis favors deposition of Aβ in cerebral vessels. Indeed, neuropathological studies showing that neuroinflammation is secondary to deposition of Aβ argue against the notion that inflammation promotes Aβ deposition [13, 14]. Thus, the cumulative amount of evidence implies that angiitis in ABRA is secondary to deposition of Aβ in vessels. Already, the initial report on ABRA has already pointed out that angiitis may be due to vascular Aβ [15]. Subsequent studies have shown that inflammation is specific to vessels with significant Aβ deposition [8, 16]. Furthermore, autoantibodies to Aβ can be observed in cerebrospinal fluid and are produced by expanded B cells derived from an ABRA patient [17, 18]. Finally, ABRA seems to occur spontaneously in a murine model with excessive production of vascular Aβ [19]. Interestingly, recent therapeutic trials in AD, with active immunization against Aβ as the mechanistic principle, have drawn attention to ABRA. In patients immunized against Aβ, occurrence of specific antibodies seems to go along with clearance of Aβ from brain parenchyma, immune activation and vascular inflammation [20–22], as well as potentially more prominent vessel wall deposition of Aβ [23]. Therefore, in order to explore whether enhanced clearance of parenchymal Aβ occurs in ABRA and to assess whether enhanced clearance of Aβ correlates with parenchymal immune activation, we carried out a case control study. For this, 11 patients with histologically proven ABRA and 10 patients with CAA were chosen. To exclude bias towards enhanced Aβ loads found in CAA with AD [2, 24], we recruited ABRA and CAA patients according to identical criteria with no reference to cognitive status. We could show that ABRA patients harbor significantly fewer plaques in cerebral parenchyma and show decreased Aβ loads compared to CAA controls. This reduction of plaques corresponds with a significant increase in activated macrophages and higher contents of microglia. This is in line with the hypothesis that ABRA results from Aβ-triggered immune activation.

Methods

Patients and Controls

Brain tissue specimen of 11 patients with ABRA (9 from diagnostic biopsies and 2 from autopsies) and 10 patients with CAA (only diagnostic biopsies) were collected in Northern Germany from 2002 to 2011. For biopsy samples, vascular events leading to diagnostic intervention were used as entry criteria to this study. The 2 autopsy patients died acutely due to ABRA-related events. Patients with clinical signs indicative of dementia or suspected AD were specifically excluded from this study. Selecting patients with vascular events in the absence of dementia may have introduced a bias towards severe forms of CAA, yet bias towards elevated levels of parenchymal Aβ, which strongly correlates with dementia, was avoided. The use of specimens and basic clinical information was in agreement with the regulations and ethical standards of the contributing hospitals and written consent by patients or relatives was obtained when appropriate.

Neuropathological Investigations and Immunohistochemistry

Paraffin-embedded tissue samples were cut into 3-μm thick-serial sections, mounted on glass slides and processed according to published protocols [25]. Besides hematoxylin-eosin, immunohistochemical stainings with the following primary antibodies were performed: Aβ (1:100; Mob410; DBS Emergo [26]), tau (1:1,500; Thermo), human leukocyte antigen-DR (HLA-DR; 1:100; M0775, Dako), CD68 (1:50; 2164; Immunotech). Primary antibodies were visualized using a standard diaminobenzidine streptavidin-biotin horseradish peroxidase method (for Aβ, tau and HLA-DR; Ventana/Roche) or an alkaline phosphatase method (for CD68; Ventana/Roche). Quantification of immunosignals was performed according to published methods [27] by experienced morphologists (S.B. and M.G.) blinded with respect to the experimental groups. Briefly, for quantification of diffuse and cored Aβ plaques, CD68-positive macrophages, HLA-DR-positive microglia and neurofibrillary tangles, we counted the presence of positive events (plaques, positive cells and tangles) in a representative fraction of the entire sample (at least 1 mm^2) using a Zeiss DMD 108 large image area microscope. For quantification of immunopositive ar-

Table 1. Demographic, imagenological and clinical features of 11 ABRA and 10 control (CAA) patients

	Age, years	Average	Sex	Main symptom	Neuroradiological findings	Site of biopsy[a]
ABRA	70	67.5±5.2	F	subacute confusion	multifocal leukoencephalopathy	n.a.
	67		M	grand-mal seizure	n.a.	n.a.
	69		M	aphasia, apraxia, dementia	temporal mass lesion	temporal
	73		F	hemiparesis, progressive dementia	occipital mass lesion	temporal
	61		M	headache, confusion	multiple vascular-like lesions	frontal
	63		M	n.a.	parietooccipital mass lesion	parietal
	78[a]		F	confusion	leukoencephalopathy	occipital
	60		F	n.a.	n.a.	n.a.
	66		M	n.a.	n.a.	n.a.
	68		F	grand-mal seizure	encephalitis-like pattern	n.a.
	68[a]		M	progressive confusion	n.a.	frontal
CAA	72	68.6±6.0	F	n.a.	intracranial hemorrhage	frontal
	74		F	n.a.	intracranial hemorrhage	frontal
	68		M	n.a.	intracranial hemorrhage	frontal
	63		F	n.a.	intracranial hemorrhage	frontal
	67		M	n.a.	subdural hygroma	n.a.
	76		M	n.a.	n.a.	frontoparietal
	67		F	acute aphasia	intracranial hemorrhage	frontal
	77		F	n.a.	n.a.	parietal
	56		F	n.a.	intracranial hemorrhage	n.a.
	68		M	n.a.	intracranial hemorrhage	temporal

n.a. = Not available. [a] Autopsy.

eas (Aβ, tau, CD68 and HLA-DR) fifteen randomly chosen 0.2-mm² regions of each sample were assessed using Zeiss AxioVision quantification software on images taken with a Zeiss Axiovert S100 microscope. Only parenchyma at least 200 μm distant from sites of angiocentric inflammation or vessels was included in the quantification.

Statistical Analysis

The data were analyzed using SPSS 17 statistical software (SPSS Inc., Chicago, Ill., USA). Analyses included Kolmogorov-Smirnov and Shapiro-Wilk tests for normal distribution assessment. For comparison of means, Student's t test was applied for two groups if they were normally distributed. Statistical significance of all analyses was determined at $p < 0.05$.

Results

Description of ABRA and CAA Cohorts

Details on the two patient groups are given in table 1. Within the ABRA patients (5 female, 6 male, average age 67.5 years, standard deviation, SD, 5.2 years) the principle presenting complaint was acute or subacute confusion. Neuroimaging and biopsy (or autopsy for the 2 individu-

als who died as a consequence of ABRA; for sites of biopsies see table 1) were performed to establish the diagnosis of cerebral angiitis. The CAA patients (6 female, 4 male, average age 68.6 years, SD 6.0 years) presented with intracerebral hemorrhage without distinct signs of dementia. Neuroimaging and eventually biopsy (for sites of biopsies see table 1) were performed to establish the diagnosis of CAA. The diagnosis of ABRA was made histologically according to current diagnostic criteria with prominent transmural lymphocytic and granulomatous infiltrates, mainly in smaller arteries (fig. 1) [5]. Deposits of Aβ could be seen in a substantial number of vessels. Notably, angiitis could only be observed in vessels with Aβ. In the CAA samples, well-defined Aβ deposits in the vessel walls were abundant. However, no signs of inflammation could be seen in Aβ-containing vessels (fig. 1).

ABRA Patients Have Less Aβ Plaques and Increased Immune Activation in Parenchyma Compared to CAA

We were interested to see whether Aβ plaques or Aβ loads in ABRA patients differ from those found in CAA

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controls. To this end, we counted Aβ plaques (diffuse and cored) and determined Aβ loads in brain parenchyma distant from sites of vascular inflammation. ABRA patients harbored significantly less Aβ plaques than CAA controls (16.2 ± 4.4 Aβ plaques/mm^2 for ABRA; 51.5 ± 11.4 Aβ plaques/mm^2 for CAA controls, p = 0.013), whereas for Aβ loads we detected a nonsignificant reduction in ABRA patients (1.2% ± 0.4/area for ABRA; 2.1% ± 1.1/area for CAA controls, p = 0.44; fig. 2). This means that reduction in plaque load is more drastic than reduction in diffusely deposited Aβ. In contrast, tau pathology was comparable in both cohorts by counting tau-positive neuropil threads or when quantifying brain parenchyma showing tau positivity (0.31% ± 0.11/area for ABRA; 0.20% ± 0.11/area for CAA controls, p = 0.51; 28 ± 11 tau-positive neuropil threads/mm^2 for ABRA; 11 ± 14 tau-positive neuropil threads/mm^2 for CAA controls, p = 0.68).

Since reduced Aβ burden has been shown to correlate with enhanced immune activation, and ABRA patients have been shown to mount an immune response to Aβ, we assessed immune activation in ABRA patients and CAA controls [13, 14, 17, 18]. We counted CD68-positive macrophages and HLA-DR-positive microglia in brain parenchyma distant from sites of vascular inflammation and determined percentages of brain parenchyma showing positivity for the above-mentioned markers. ABRA patients showed significantly more brain parenchyma with CD68-positive immunosignals and more CD68-positive macrophages than CAA controls (0.98% ± 0.24/area for ABRA; 0.24% ± 0.05/area for CAA controls, p = 0.015; 100 ± 60 CD68-positive macrophages/mm^2 for ABRA; 29 ± 23 CD68-positive macrophages/mm^2 for CAA controls, p = 0.31; fig. 2). Regarding HLA-DR-positive microglia there was no significant difference between groups (1.42% ± 0.37/area for ABRA; 1.01% ± 0.47/area for CAA controls, p = 0.50; 84 ± 24 HLA-DR-positive microglial cells/mm^2 for ABRA; 58 ± 11 HLA-DR-positive microglial cells/mm^2 for CAA controls, p = 0.33).

Macrophages Engulf Aβ in ABRA

In view of the assumption that the immune response in ABRA might be directed against Aβ, we performed immunohistochemical double-labeling using antibodies directed against CD68 and antibodies directed against Aβ in biopsies of ABRA and CAA patients. Only in ABRA patients could we observe CD68-positive macrophages in the immediate vicinity of Aβ plaques and vascular Aβ (fig. 3). We frequently found CD68-positive macrophages engulfing Aβ both in vessel walls (fig. 3a) and within the parenchyma at edges of Aβ plaques (fig. 3b).
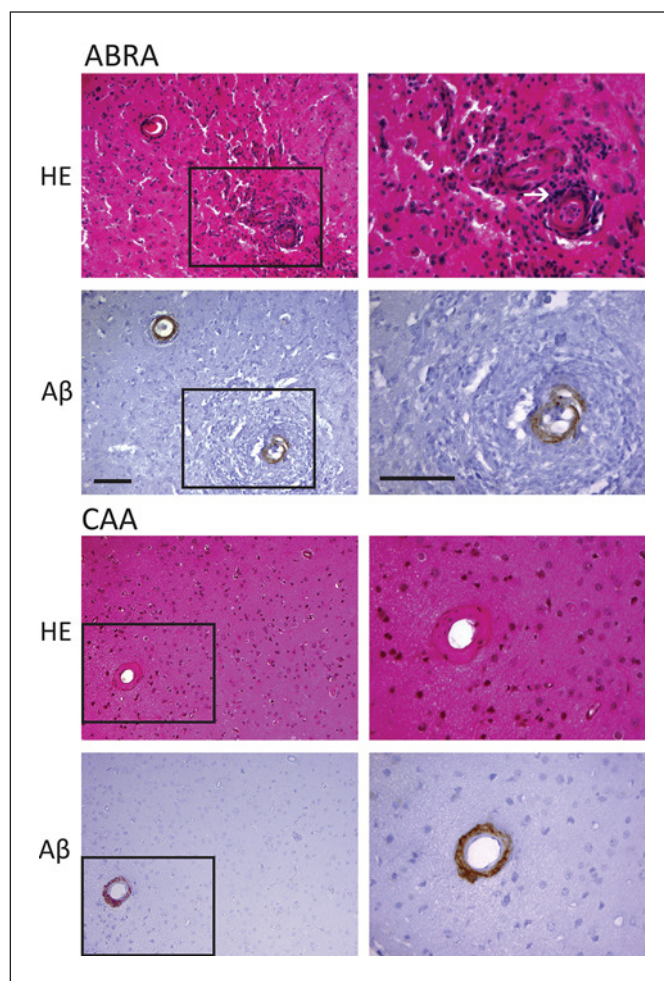
Fig. 1. Representative HE and Aβ staining of ABRA (top 2 rows) and CAA (lower 2 rows) patient samples. Vessel walls show Aβ deposits in ABRA and CAA (rows 2 and 4). Inflammatory cells including the presence of giant cells (arrow) can only be seen in vessels of ABRA patients where Aβ deposits are prominent (rows 1 and 2). Right panels show magnification of indicated regions. Scale bar = 100 μm.

Discussion

Here we histologically analyzed the largest series of biopsies from ABRA patients to date [9]. ABRA is thought to be caused by an inflammatory response to Aβ deposited in vessel walls [5, 17, 18, 28]. The objective of this study was to investigate whether the inflammatory response to Aβ has any effect on the status of immune activation in the brain parenchyma at sites distant from vessels. Furthermore, we wanted to investigate whether this neuroinflammatory response would correlate with enhanced clearance of Aβ in ABRA. Our study clearly shows
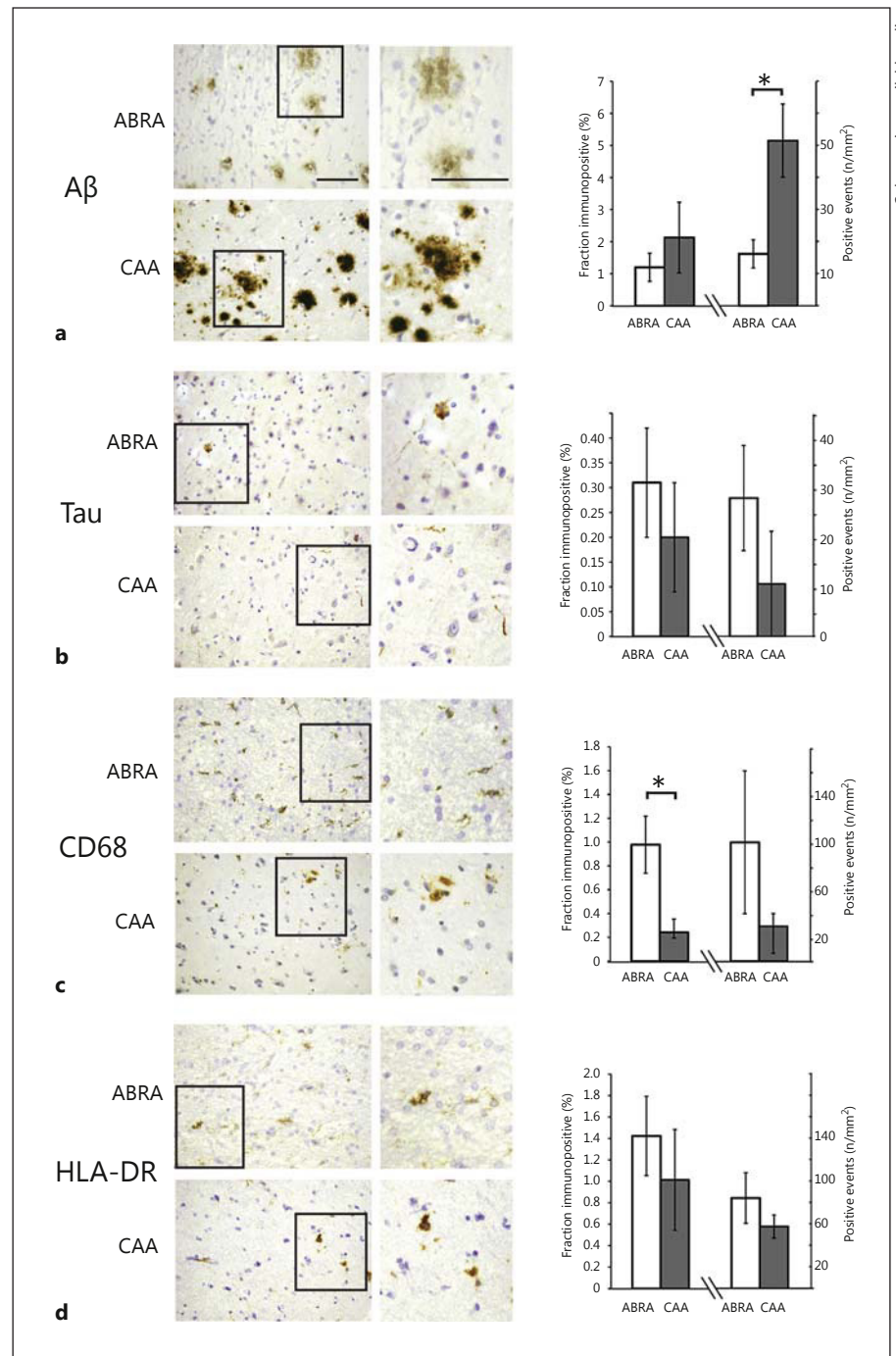
Fig. 2. Quantification of immunohistological staining for Aβ, tau, CD68 and HLA-DR. Representative histological images of immunohistological stainings for Aβ (**a**), tau (**b**), CD68 (**c**) and HLA-DR (**d**) are shown with magnification of indicated regions. Both positive events (plaques, positive cells and tangles) and percentages of immunopositive areas were quantified separately and are shown as group averages for percentages of immunopositive areas (left part of the graph) and positive events (right part of the graph). * p < 0.05, indicates significance (Student's t test). Scale bar = 100 μm.

that neuroinflammation in ABRA is not limited to affected vessel walls but also extends to the surrounding parenchyma with significantly more CD68-positive macrophages. This translates into a reduced Aβ plaque burden in these patients. We could also observe CD68-positive macrophages surrounding Aβ plaques and engulfing Aβ

in ABRA but not in CAA controls, implying that the inflammatory response to Aβ triggered by vascular deposits of Aβ may lead to active clearance of parenchymal Aβ.

Why are these findings relevant? Over the last decade, ABRA has been recognized as a well-defined disease entity that represents a substantial subset of PACNS [4–6].
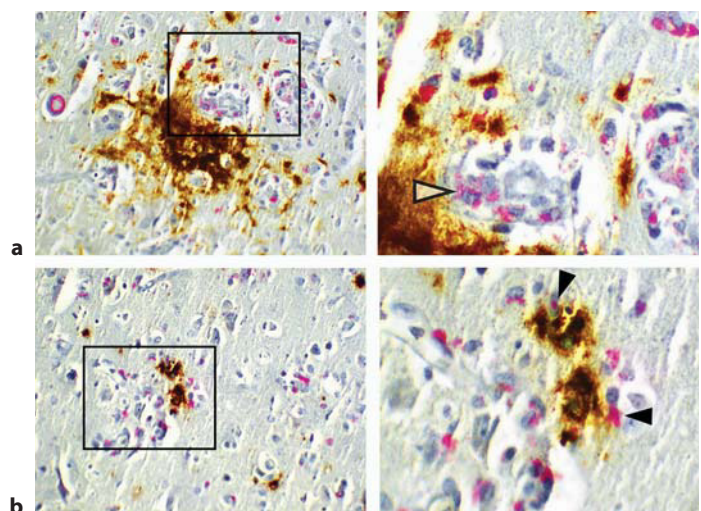
Fig. 3. Immunohistochemical double-labeling for Aβ (brown; color refers to online version only) and CD68 (violet) of ABRA samples. CD68-positive activated macrophages engulfing Aβ-positive material can be found at the site of perivascular Aβ deposits (**a**, unfilled arrowhead) and in the immediate vicinity of plaques in the cortical parenchyma (**b**, solid arrowheads).

The pathophysiology of ABRA is not entirely understood, but the most likely etiology is excessive immune response to vascular Aβ [17, 18, 28]. Our data are in line with an immunological origin of ABRA and suggest that the immunological response to Aβ extends into the brain parenchyma with the potentially beneficial effects of an enhanced clearance of Aβ.

There is an ongoing discussion on the presence of Aβ and tau deposits in ABRA. While the majority of studies found intermediate levels of Aβ in ABRA, the presence of tau is reported less frequently [5, 29]. The fact that we found comparable, albeit low, amounts of tau in ABRA and CAA controls shows that tau deposits may be more frequent in ABRA than previously thought. We are confident that the observed decrease in parenchymal Aβ is not the result of a systemic error due to incorrect selection of patients. We specifically recruited patients to the CAA control group on the basis of vascular events leading to diagnostic intervention, with none of the patients presenting with obvious signs of AD-related dementia. The dissociation between comparable tau loads and significantly lowered Aβ loads in ABRA patients also speaks in favor of selective clearance of Aβ but not tau. Similar dissociation could be observed in patients with active immunization against Aβ [22, 30, 31]. Neuroradiological abnormalities have been reported in patients treated with monoclonal antibodies to Aβ [32]; these point to a correlation between neuroradiological findings indicative of vascular pathology and amyloid clearance. Our data show that ABRA patients with histologically proven inflammatory vascular pathology have enhanced amyloid clearance, further highlighting similarities between ABRA and Aβ antibody-based therapies [33]. Taken together, our findings support the ever-growing evidence that autoimmune dysregulation, characterized by an adaptive Aβ-directed immune response, plays a critical role in AD in general, but specifically in ABRA where vascular Aβ deposition may act as the triggering event [13, 18, 28].

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Kontext der Publikation und weitere Ergebnisse der Dissertation
Context of the publication and further results of the dissertation

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Dementia and Alzheimer's disease

Dementia is a frightening disorder. We fear it for the diffuse, permanent damage it appears to do to the essence of a human being. Only very limited therapeutic or preventive options are available. Dementia is strongly correlated with age and a major public health concern. The prevalence of dementia is estimated to be greater than 5 % in people older than 60 years in the western world (Ferri et al. 2005) and sometimes reported to exceed 25 % in the over 90 year-olds (Qiu et al. 2009, Reitz et al. 2011).

Dementia is a descriptive clinical term for a multiform syndrome whose causative mechanisms are insufficiently understood. Next to vascular degeneration, Alzheimer's disease (AD) has been identified as the main entity leading to dementia (Ropper and Samuels 2009). In popular use "Alzheimer" often even acts as an umbrella term for any age-related cognitive impairment.

The diagnosis of AD is made by clinico-pathological observation, namely the concurrence of progressive cognitive impairment - first memory-related, then wide-spread - with changes in histology and anatomy: 'Senile plaques' in the brain parenchyma, neurofibrillary tangles within neurons and substantial brain atrophy. Senile plaques are organized around a core with high concentration of β-amyloid (Aβ), a 40 to 42 amino acid-long peptide that has oligomerized in beta-sheets which in turn organize as fibrils.

Until recently, definite diagnosis by quantification of Aβ plaques could only be made post-mortem. This may be about to change with the development and validation of PET in-vivo imaging techniques (Klunk et al. 2004, Lister-James et al. 2011).

The exact role of Aβ in a mechanistic cascade that leads to neural damage is not fully understood.

The main questions that still wait for a definite answer are i) if Aβ accumulation is causative and necessary for neural degeneration and ii) if so, what causes this accumulation in the first place?

Question i) is commonly referred to as the " Aβ-hypothesis". Strong evidence exists in favor of it, a definitive answer from preventive intervention trials seems to be within reach. Recent findings such as the prion-like propagation of condensed Aβ (Ashe and Aguzzi 2013) and a Aβ-lowering mutation providing protection from cognitive impairment (Atwal et al. 2012) may give new insight into the disease mechanisms.

Question ii) is a very difficult one since slow processes related with ageing on a whole body system level need to be considered, e.g., the role of inflammation, the interplay with the immune system and with the vascular component of the brain.

However, doubt exists if Alzheimer's disease can be fully understood in the framework of a reductionist search for a single cause. Many mechanisms on a system level might interact in a network manner - rather than like in a linear chain of events - over very long time scales and then lead to "Alzheimer syndrome", as Richards and Brayne provocatively term it when they ask "What do we mean when we say Alzheimer's disease"? (Richards and Brayne 2010).

The present dissertation consists of two projects.

Firstly, leading to the present publication, we focused on aspects of Aβ-clearing in the context of "Aβ related angiitis" (ABRA), an Aβ-related inflammatory disorder of the brain vasculature. It had been suggested that in ABRA, an immune reaction to vessel wall Aβ is the trigger of vasculitis and often encephalitis. We present the quantitative immuno-histochemical analysis of eleven new cases against controls with a focus on parenchymal immune activation and Aβ load. Our results support the hypothesis of an inflammatory component in ABRA, which extends into the cortical parenchyma and which is directed against Aβ. ABRA may thus help to inform Aβ -immunization strategies against Alzheimer's disease.

Secondly, we built a cell model for an important Familial Alzheimer's disease (FAD) mutation and started to test hypotheses of non-Aβ toxicity in this model. For both projects, resources had been built in our research group over some years. For ABRA, a rare, relatively recently established disease entity, pathology samples were collected over several years by neuropathology institutes throughout Northern Germany in order to construct the largest case collection to date, amenable to statistical evaluation well beyond the scope of case reports. In the case of Familial AD, there has been a long-standing link of our group with research and health care centered around a community in Colombia with high prevalence of the distinct E280A familial Alzheimer disease mutation. This collaboration extends from the exchange of senior researchers to the access to high quality autopsy samples. The E280A Alzheimer variant in Colombia causes enormous suffering in the community and has recently attracted international attention with the high-profile 'Alzheimer Prevention Initiative', an antibody based early-intervention trial, that has the potential to provide relief and definitive evidence in favor of the Aβ hypothesis (Reiman et al. 2012).

Background: The role of Aβ, disrupted homeostasis and abundance
The definition of Alzheimer's disease has changed little over more than 100 years. Clinically observed progressive cognitive impairment - first memory-related, later generalized - is correlated with the histo-pathological hallmarks of plaques and tangles. Brain volume decreases with duration and severity of Alzheimer's dementia (Fotenos et al. 2005).

Plaques are extracellular conglomerates made up of a large number of different proteins. In their center, they contain large amounts of aggregates of a certain beta-sheet folded 5 kDa protein, ranging from oligomers to large fibrils of many thousand proteins. The plaque is susceptible to beta-sheet amyloid staining techniques like the Congo-red or the Mann stain, used by Alois Alzheimer in his early discovery work. Figure 1 shows a sample of plaque-laden cortex of one of his first patients, not of Auguste Deter, his often-cited index-patient, but of a 54 year-old man who had died in 1910 at the end of a few years of rapidly progressing dementia (Alzheimer 1911). The patient at the time died eventually of pneumonia, which is still today typical of late disease when swallowing becomes impaired. It has been estimated that AD shortens life expectancy by about ten years. Figure 2a shows a large plaque stained immunohistologically in our laboratory. It also shows deposits of the beta-amyloid or Aβ inside neurons (arrowheads) and, quite typically, within the walls of blood vessels.
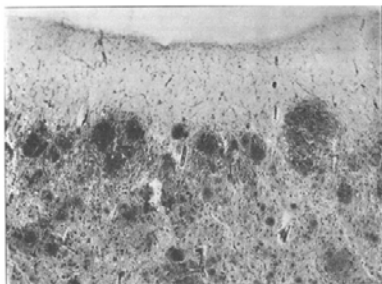

Fig. 1: Amyloid-beta plaques as described by Alois Alzheimer in the original publication (Alzheimer 1911).
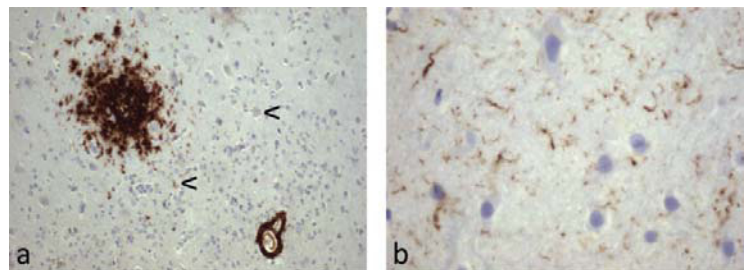
Fig. 2: a) Amyloid-beta neuritic plaques intraneuronal diffuse amyloid deposits (arrowheads). b) tau tangles. Both stainings from our laboratory.

Large plaques are commonly referred to as neuritic plaques, in contrast to the 'diffuse' intracellular deposits of Aβ, see Figure 2b.

Although cases of fully intact cognition until death in the presence of large Aβ loads at autopsy are not rare, the correlation of post-mortem findings of large amounts of Aβ and the clinical picture of AD is very good (Berg et al. 1998). What is Aβ and is its accumulation due to to over-production or insufficient clearing? We first turn to production.

Aβ Production: APP, the gamma secretase and presenilin

Enormous research effort over decades has been directed at the elucidation of Aβ production (e.g. Strooper and Annaert 2010). In short, Aβ is the product of neuronal intra-cell membrane cleavage of a much larger precursor protein, the 110-135 kDa APP, by the sequential action of two distinct hydrolysing enzymes or enzyme complexes, the beta- and gamma-secretases. APP stands for amyloid precursor protein, a name indicating that the protein had not been

described before in another function. The secretases meet their substrate in lipid raft regions of the cell membrane (Strooper and Annaert 2010) and Aβ is secreted into the extra-cellular space. While the beta secretase predominantly cleaves at a unique site, the resulting 99 amino-acid carboxy-terminal fragment is cleaved by the gamma secretase with some variability. The resulting Aβ protein ranges from 38 to 43 amino-acids, with a predominance in size of 40, followed by 42 amino acids. The exact balance of the different Aβ types is being heavily investigated and it is implied in AD disease mechanisms. Aβ-42 is more hydrophobic and prone to condensation than Aβ-40. It is often described as the most toxic variant (Pauwels et al. 2011).

The beta secretase (or BACE) has until recently attracted only limited attention. We, too, focus here on the gamma secretase.

The gamma secretase (γ-secretase) is a multi-protein complex, consisting of presenilin, nicastrin, aph-1 and pen-2. Presenilin is the catalytic subunit.

There are two variants of Presenilin, encoded by different genes: presenilin 1 (PSEN1 on chromosome 14) and presenilin 2 (PSEN2 on chromosome 1). In the human brain presenilin 1 expression is dominant as compared to presenilin 2 (Lee et al. 1996). Presenilin is a 47 kDa, 467 amino-acid transmembrane protein with nine intra-membrane domains. The catalytic site lies between in the sixth and seventh transmembrane domain and is encoded by exons 7 and 11 (out of 12 exons).

Familial Alzheimer's disease

Presenilin plays an important role in AD research as the key protein in 'Familial Alzheimer's disease' (FAD). Onset in FAD is several decades earlier as compared to old-age related AD (which is referred to as 'sporadic', since it does not run in families, while making up more than 99 % of the burden of AD).

Today, FAD has been linked to more than 200 point mutations in the PSEN1 gene and about 30 mutations of the APP encoding (alzforum 2014). Heterozygous mutation leads to the phenotype.

In view of the dominant transmission mode, the idea of a gain of function in the gamma cleavage step as the reason for Aβ overproduction is obvious. Either the cleaving enzyme or the substrate might show increased efficiency and/or affinity.

Given that the very last step of Aβ production is affected by the point mutation associated with the full AD phenotype, Aβ abundance seems very much to be causal for the neurodegeneration of AD.

It is noteworthy that also *somatic* PS1 mutations lead to an AD phenotype. A spectacular case has been diagnosed in 2013 with a delay of 107 years from DNA in original brain tissue

samples: Auguste Deter, Alois Alzheimer's index-patient, harboured a somatic presenilin mutation (Müller et al. 2013).

In the broader scheme, one hopes to learn general lessons from FAD: "… Presently the FAD mutations are the only identifiable causative agents of AD and these mutations may offer the best available models for the study of the cellular and molecular mechanisms involved in the development of the more common sporadic disease. Since the clinical manifestiations and neuropathology seems similar in both sporadic and familial AD, lessons learned from studying the mechanisms of FAD should also be applicable to sporadic AD." (Robakis 2010).

Aβ clearance

Reduced clearance could disrupt homeostasis just as well as overproduction.

Of high relevance is Apolipoprotein E (ApoE) which acts as a chaperone and transporter of Aβ. Three variants of this protein are common, ApoE2, ApoE3 and ApoE4. ApoE4 is by far the single most important genetic risk factor for developing AD in a dose dependant manner for hetero- versus homozygous carrier (Hauser 2013). ApoE2 is moderately protective against AD. ApoE overexpression has been shown in a mouse model to enhance clearance of Aβ and reverse cognitive deficits (Cramer et al. 2012). Two of the main Aβ degrading enzymes, neprilysin and IDE (insulin degrading enzyme) are regulated by ApoE. Since neprilysin and IDE levels in vivo are correlated with aging, the potential role of decreased Aβ degradation is attracting more and more attention (Mawuenyega et al. 2010).

Immune activation

Not very much is known about the role of the immune system in Alzheimer's disease. This seems out of proportion with the complexity of the immune system and its prime importance in the human body. In many chronic disease entities, the immune system has eventually emerged as a key factor (Pawelec et al. 2014, Vasto et al. 2009).

Here, we would like to cite three aspects of the interaction of the immune system with Aβ that highlight the diversity of the interplay.

Firstly, the role of microglia in the clearance of Aβ. The brain monocytes react to plaque formation and are critical in controling their size (Meyer-Luehrmann et al. 2008, Simard et al. 2006). Microglia dysfunction has been linked to decreased Aβ clearance (Streit 2004).

Secondly, a genetic defect in anti-inflammatory function of myeolid cells is strongly associated with AD (Jonsson et al. 2013).

Thirdly, antibody-dependent clearance of Aβ is tested as therapy and prevention for AD, see above. Important questions are: Does antibody cross the blood brain barrier? Which form of Aβ is opsonized, and where? In the extra-thecal blood stream or intra-thecally? What are the

side-effects of active and passive immunization? Which other immune mechanism are triggered by introducing anti-Aβ antibody?

In comes Aβ related angiitis (ABRA) as a rare cerebral vasculitis that is thought to be triggered by auto-immune activation against Aβ deposits in cerebral vessel walls.

It is natural to see in ABRA a model system for immune action against Aβ from which one might learn about some of the above question.

Our contribution:

A) Immune activation targeting Aβ in a case series of ABRA

This project lead to the present publication. We looked at a collection of cases of Aβ-related angiitis (ABRA) which we compared to a carefully chosen control group. We were interested in evidence with respect to the hypothesis of an Aβ-triggered immune reaction as the pathophysiological mechanism in this disorder. In particular, we looked at immune activation, Aβ clearance and the presence of specific antibody.

It is the largest series at the time of publication of this increasingly diagnosed, rare condition. The series samples were uniformly processed and allow for statistical analysis unlike a miniseries or a review of a number of the many individual case reports in the recent literature. Summarizingly, we found evidence for our hypotheses and refer to the original text of the publication above.

Additional evidence that was not included in the publication comes from a TAPIR assay (tissue amyloid plaque immuno-reactivity, Kellner et al. 2009). It tests for plaque-specific antibody in the CSF and serum of ABRA patients. We had access with consent to pairs of CSF and serum of two patients. In TAPIR, samples of plaque-loaden transgenic mouse brain is incubated with patient serum and specific antibody binding is visualized by anti-human-IgG fluorescent secondary antibody. Binding could be demonstrated, see Figure 3. The sample size, however, was too small to draw strong conclusions.
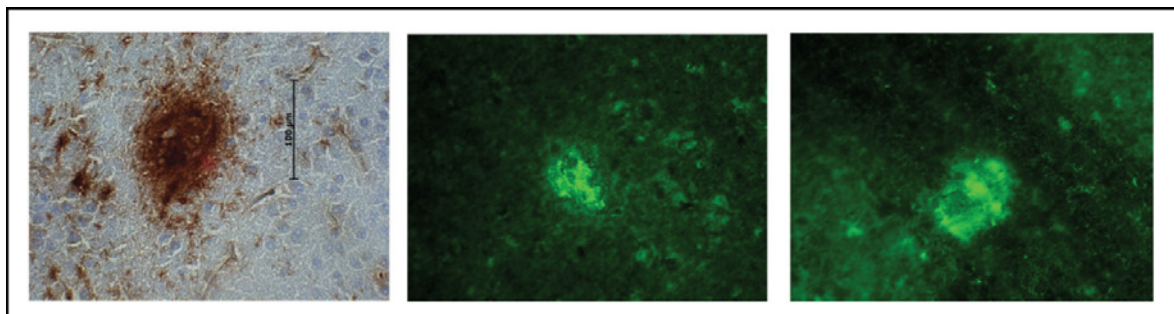

Fig. 3: TAPIR assay: Murine amyloid-beta plaques, immunohistochemistry with amyloid antibody (left) and decorated with antibody in patient serum (middle and right).

B) A cell model of an important variant of FAD

Our group has a long-standing cooperation with medical researchers in Antioquia/Colombia, where a large community with high prevalence of Familial Alzheimer's disease with the E280A presenilin mutation lives. For the many hundred affected families in the remote mountainous region the burden of early onset AD is enormous. Recently, this E280A mutation has gained prominence as the focus of AD antibody intervention shifts to very early prevention (Reiman et al. 2011).

Scientists from the region in Colombia in our group work with tissue samples from Antioquian patients and many original findings have been published (Sepulveda-Falla et al. 2011, 2012, 2014).

A cell model of the E280A presenilin mutation is clearly useful in order to complement patient sample studies with in vitro experiments. We built this cell model and tested one first hypothesis of a Aβ-independent pathomechanism, namely impaired response to endoplasmic reticulum stress, in the new model.

Gene constructs

Copy–DNA of presenilin was bought from a commercial supplier and transfered from the shuttle vector into our expression system of choice. The point mutation E280A (glutamic acid for alanine at amino acid 276) was then introduced and all products were checked by sequencing.

In FAD with its clear point mutation cause one of the few established animal models is the presenilin Exon-9-deletion (ΔE9) mouse. The exon 9 deletion is a variant of human FAD where a point mutation causes mis-splicing and deletion of one exon of 87 nucleotide/29 amino acids. The resulting phenotype is early onset AD with typical Aβ-plaque overload both in humans and mice. This variant of FAD often acts as a common reference point for cell culture and mouse experiments in the field of FAD. It allows to compare across mutations and to relate cell line and mouse findings. We hence tried to add this variant to our cell lines and have the gene construct ready in an expression system after some intricate cloning.

Cell lines

Different cell types were considered: HEK293 human embryonic kidney cells are robust, proliferate fast and are relatively easy to transfect. SH-SY5Y, a human neuroblastoma derived cell line, has the great advantage of being of the neuronal type, these cells, however, are much more delicate to handle and more difficult to transfect. Both of these cell lines have endogenous PS1 expression, which does not rule out these cells as a model system, since FAD is a heterozygous point mutation disease. In order to study the pure effect of a mutated PS1 gene, one could also make use of a PS1 knock-out cell line, i.e., mouse embryonic

fibroblasts (MEF). We decided to work with all three approaches in parallel. SH-SY5Y cells were first obtained as a kind gift from colleagues at a neighbour institute, later however purchased in order to have control of the passage number in this delicate cell line. MEF cells were obtained as a gift from P. Saftig, Kiel.

Transient transfection were successfully tested with the liposomal transfection agent lipofectamine 2000, which was also used in building the stable cell lines.

Our goal was to build stable cell lines with PS1 wild type and PS1 E280A overexpression of moderate and approximatively equal magnitude, see Figure 5.

This was achieved for the HEK293 cells in a dilution technique in order to be able to grow cultures of monoclonal origin.

After transfection, the non-adherent cells were resuspended in a series of increasing dilution and cultured. Eventually, culture wells were chosen where colonies had clearly grown under selection pressure from a single clone.

Appropriate selection pressure was determined beforehands to be 400 µg Zeocin in standard fetal calf serum enhanced DMEM. Clones were expanded and frozen in sufficient quantity as to be able to start dozens of experiments from the same passage number.

Monoclonal origin of a cell line is important. A polyclonal culture that had taken up the E280A construct as confirmed by PS1 over-expression (by western blot) lost the PS1 E280A gene over time, while retaining the Zeocin resistance.

In multiple attempts, SH-SY5Y could not be transfected in a stable manner. Toxicity of the transfection agent, selection cytotoxicity and the loss of cell-cell contact showed to be too strong in our setting for the vulnerable neuronal cells. The most promising attempt was done in reverse transfection technique, where plates are coated with DNA-laden liposomes before to-be transfected cells are added, which are thought to then literally sit on top of the transfection liposomes.

A protocol for transient transfection of MEFs has been established by my senior colleague Alvaro Barrera.

Endoplasmic reticulum stress in the E280A FAD cell model

Small amounts of newly synthesized protein that do not pass quality checks are taken care of by endoplasmic reticulum associated degradation (ERAD) (Brodsky 2012). If ERAD is overwhelmed, the accumulation of misfolded protein in the ER triggers a physiological three-axis coping program called the 'unfolded protein response' (UPR) which enhances degradation but mainly works by the reduction of protein metabolism (Chakrabarti et al. 2011).

The unfolded protein response has been implicated in non-familial AD pathophysiology as an early event in the slow process of neurodegeneration and a trigger of inflammation

(Hoozemans et al. 2012, Hoozemans and Scheper 2012, Roussel et al. 2013, Lindholm et al. 2006, Salminen et al. 2009).

A cluster of publications ten years ago reported UPR deficiencies of familial AD PS1 mutant-carrying cells (Katayama et al. 1999, Katayama et al. 2004), rendering cells more vulnerable to many kinds of adverse conditions (Terro et al. 2002, Yasuda et al. 2002). Blocking of one of the intra-luminal sensors of ER stress by mutated PS1 was cited as a potential mechanism. These results were not confirmed unanimously (Sato et al. 2000). However, anomalies in ER stress response protein expression was also found in our Colombian brain samples (Sepulveda-Falla 2010).

This and our group's interest in 'beyond-amyloid cascade mechanisms' in AD plus the expertise in ER stress response from our group's research lines on prion disease and neuroserpin motivated us to test our E280A cell model under ER stress.

In order to induce stress, we used tunicamycin, a glycosyl-transferase inhibitor, that causes accumulation of protein in the ER. BiP binds unfolded protein and delivers it to the stress sensors IRE1α, PERK and ATF6, see Figure 4. Our read-outs were both the intra-ER-luminal protein BiP, the sensors and the downstream transducers, notably eIF2α (eucaryotic intiation factor), which is phosphorylated by PERK if stress is sensed.
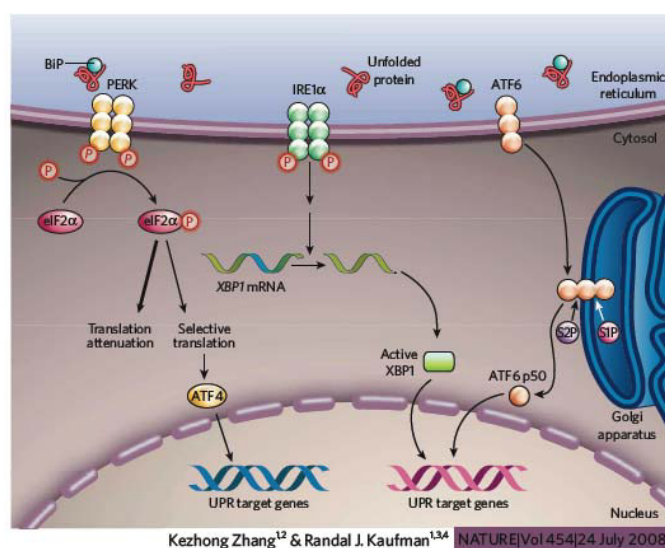

Fig. 4: Three pathways of the unfolded protein response (UPR),
from (Zhang and Kaufman 2008).

Non-transfected HEK cells, wild-type PS1 and E280A PS1 HEK cells were grown to 90 % confluency in T75 flasks and exposed to tunicamycin at a concentration of 50 ng/ml which we added to standard cell culture medium (DMEM + 10 % FCS). Zeocin as the selection agent for the stable cell lines was not added during the application of ER stress. After 20h, cells were harvested. At this time point, the E280A cells started to show gross morphological

changes. Cell lysate of these three stress samples together with three corresponding non-stressed cell lysates was separated by SDS PAGE and analysed by western blot.
The experiment was repeated four times. Input gross protein for each lane in the gel was equal within errors of the BCA protein quantification assay and all quantifications were normalized to actin.

The logic of intervention and control cells in this experiment is the following: untransfected cells are compared under exactly the same stress conditions with the transfected ones as to establish the base-line deviations introduced by transfection. The real test of any hypothesis pertinent to the point mutation E280A is the comparison of wild type PS1 transfected versus E280A transfected cell lines, see Figure 6 for a western blot of a typical experiment .

The presenilin over-expression model does not induce baseline ER stress as measured by BiP expression

Quantification of absolute and relative BiP response to ER stress is shown in Figure 7, the induction of ER stress is clearly demonstrated. The differences in BiP expression under stress between the cell lines are not statistically significant.

Stable transfection with a gene in a plasmid expression vector creates an artificial over-expression situtation. In the context of ER stress this might appear dangerously unphysiological as over-expression of any protein could possibly introduce ER stress in the first place.

Figure 7 shows that without tunicamycin-induced protein accumulation in the ER, transfected cell behave similarily to the untransfected ones. The over-expression of PS1 does not seem to induce by itself stress the response to which we would like to examine.

Our cell line is in this respect not un-physiological.

ER stress response is subdued in the PS1 E280A cells compared to PS1 wild type cells as measured by eIF-phosphorylation

Figure 8 showes the increase of phosphorylated over non-phosphorylated eIF as a multiple of the ratio without ER stress, i.e. the ratio of a ratio.

We observe a strongly subdued response to ER stress in this PERK/p-eIF pathway in the mutated E280A presenilin cells as compared to the cells transfected with wild type presenilin. The p-value of the t-test for this comparison is 0.08 at n = 4 independent runs of the experiment. The comparison to non-transfected cells is statistically less reliable since only one experiment with the inclusion of standard HEK cells was run successfully.
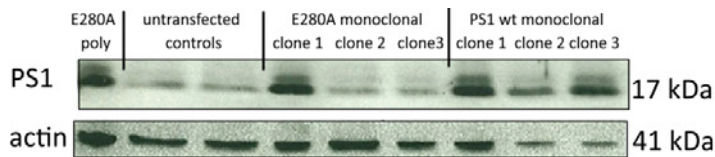
Fig. 5: Western blot of presenilin expression in selected cell populations transfected with mutated presenilin and wild-type presenilin and in untransfected controls. The transfected population were mono- or polyclonal after transfection. Constituent actin expression for normalization.
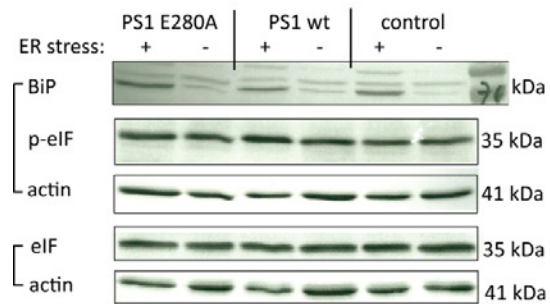
Fig. 6: Western blot of UPR protein expression for untransfected control cells, cell cultures transfected with mutated presenilin and wild type presenilin with (+) and without (-) ER stress. Actin for normalization.
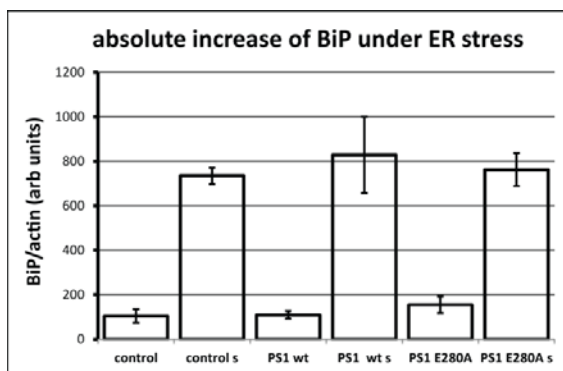

Fig. 7: BiP increase under ER stress (s) for untransfected cells (controls) and cells transfected with wild type (wt) presenilin and with mutated (E280A) presenilin; normalized to actin expression.
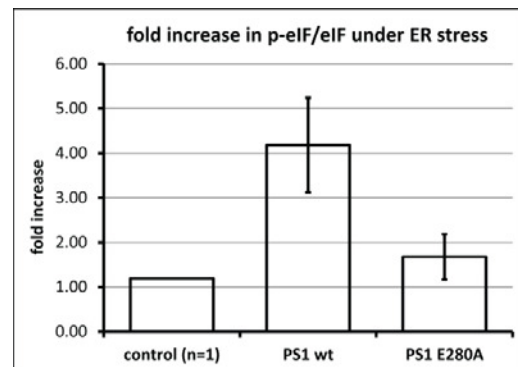
Fig. 8: Relative increase of the ratio of phosphorylated to unphosphorylated eIF under ER stress for untransfected control cells and cells transfected with wild type (wt) Presenilin and with mutated presenilin (E280A).

Summary Part B

A cell model for the important Colombian E280A FAD variant was established 'from scratch'; experiments on ER stress showed impairment of at least one of the unfolded protein response (UPR) pathways in cells carrying the mutation; not reported here, a protocol for experiments pertaining to the lipid raft expression of presenilin was established; copy DNA of the FAD presenilin variant Exon-9-deletion was built and cloned into in our expression vector of choice, ready for future use in transfections experiments.

More time would have allowed us to a) investigate the mechanism behind the subdued ER stress response of associated with the E280A mutation, b) to follow through with the lipid-raft experiments that were not mentioned here and c) to build stable cell lines of neuronal origin (SH-SY5Y) and with the Exon-9-deletion.

With the cell culture model, we hope to help our group establish a fruitful interplay of research with histological samples and cell culture in the coming years.

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Erklärung des Eigenanteils an der Publikation

Entwicklung der Fragestellung

    Die ursprüngliche Fragestellung wurde von Herrn Prof. Glatzel formuliert. Im Laufe der Arbeit wurde die Zielrichtung der Arbeit in Zusammenarbeit von Herrn Glatzel und mir weiterentwickelt, insbesondere die Wahl der zu untersuchenden Marker und Observablen und die Definition der Kontrollgruppe.

Probengewinnung und Verwaltung der Proben

    Es gelang im Rahmen der Arbeit, die bisher größte Fallzahl von Patientenmaterial mit ABRA zusammenzustellen. Die Kontaktaufnahme und Kommunikation mit den beteiligten neuropathologischen Instituten teilten sich Herr Prof. Glatzel, Herr Dr. Bernreuther und ich. Die Sammlung, Verwaltung, Lagerung und Archivierung der Proben lag in meiner alleinigen Verantwortung.

Definition und Zusammensetzung der Kontrollgruppe

    Die Definition und Zusammenstellung der Kontrollgruppe geschah in Kooperation mit Herrn Glatzel mit großem Eigenanteil, sämtliche logistischen und technischen Aspekte lagen alleine in meiner Verantwortung.

Immunohistochemische Färbung und Erstellung der quantifizierbaren Proben

    Die Erstellung der Proben und die immunohistochemischen Färbungen im Diagnostiklabor unseres Instituts wurden von mir geplant und koodiniert. Einige der speziellen Färbungen (z.B. Doppelfärbungen) wurden in Zusammenarbeit mit den Mitarbeitern des Labors angepasst, um an unserem Material erfolgreich durchgeführt werden zu können.

Quantitative Datenerhebung und statistische Auswertung

    Die Entwicklung und Anpassung der Methodik zur quantitativen Immunohistochemie wurden von mir durchgeführt, ebenso wie die eigentliche Quantifizierung und Datenverwaltung. Die statistische Auswertung wurde ebenfalls von mir alleine durchgeführt, im Anschluss vorgestellt und von dritten überprüft.

Interpretation

    Die Resultate wurden in gemeinsamer Diskussion mit Herrn Prof. Glatzel mit vergleichbaren Anteilen interpretiert.

Erstellung des Manuskriptes und der Diagramme

    Das Manuskript zur vorliegenden Publikation wurde in einer ersten Version vollständig von mir alleine erstellt und dann mit Herrn Prof Glatzel gemeinsam zur Publikationsreife gebracht. Alle Diagramme, Tabellen und Bilder wurden von mir erstellt.

Bearbeitung des Manuskriptes im Peer-review Verfahren

    Die Revisionen des Manuskriptes im Laufe des Peer-review Verfahrens wurden von Herrn Prof. Glatzel und mir in etwa gleichen Anteilen erarbeitet.

Mein ganz herzlicher Dank geht an:

Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: ....