Alzheimer's disease and the β-amyloid peptide: Aβ conformers and mechanisms of spreading

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„Alzheimer's disease and the β-amyloid peptide: Variations in plaque deposition and mechanisms of spreading"

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1. Summary

More than 100 years after the first description of Alzheimer' s disease (AD), a progressive neurodegenerative condition characterized by an amnestic memory impairment and behavioral changes, the disease is still incurable. Although a small proportion of the disease is caused by familiar inheritance through mutations in the genes *Amyloid Precursor Protein* (*APP*), *Presenilin 1* (*PS1*) and *Presenilin 2* (*PS2*), most cases appear sporadic with an old age being the major risk factor for developing AD. With a percentage of about 60-80% of total dementia numbers, AD is the most common form of dementia worldwide. As a result, the disease has a large socioeconomic burden that is considered to aggravate within the next decades due to the ageing global population. AD comprises two histopathological hallmarks: the extracellular deposition of the misfolded amyloid-β (Aβ) protein into parenchymal senile plaques and vascular cerebral amyloid angiopathy (CAA) as well as the hyperphosphorylation of the tau protein into intracellular deposits, called neurofibrillary tangles (NFTs). According to the seminal amyloid cascade hypothesis postulated by Hardy in 1992, the accumulation and subsequent aggregation of the Aβ protein into various soluble and insoluble conformations is the initial trigger of the disease. Aβ aggregation is supposed to cause further downstream effects inducing the formation of NFTs, synaptic loss and ultimately the clinical symptoms of dementia. Therefore, the Aβ peptide is of special interest in AD research. Overwhelming research has recently shown that the Aβ peptide shares similarities with the prion protein, an infectious protein that aggregates and has the ability to transmit its misfolded shape onto physiological variants of the same protein. The misfolding and ensuing propagation throughout the central nervous system gives rise to several fatal and transmissible neurodegenerative diseases. Parallels to the prion paradigm include the ability of aggregated Aβ protein to induce seeding of soluble monomeric Aβ both *in vitro* and *in vivo*. The objective of this doctoral dissertation was to further investigate the prion-like characteristics of the Aβ protein. Three studies were performed analyzing aggregate conformations in a cohort of clinical subtypes of AD, observing the nature of the initial Aβ seed triggering the disease as well as the development of new mouse models to study the spatiotemporal progression of protein aggregation throughout the brain.

Recent evidence implicates that aggregate structures of the Aβ protein display polymorphisms that may influence the clinical symptoms as well as the course of the disease. In order to examine this in more detail, the first study analyzed the structural features of amyloid plaque cores within postmortem tissue of a heterogenous cohort of more than 40 AD patients displaying both sporadic as well as familiar forms of the disease. Evaluation of the structural plaque core features was performed using a combination of conformational sensitive amyloid dyes, called luminescent conjugated oligothiophenes (LCOs). The fluorescent spectra emitted from the familial AD cases were strikingly different from the sporadic cases. Additionally, typical sporadic cases and cases of posterior cortical atrophy (PCA), a subtype of sporadic AD, displayed differences. The result that amyloid conformations are different among patient subgroups

could not be explained by biochemical data or clinical patient data. While there was no difference in plaque morphology among the three different cortical brain regions analyzed within one patient, a closer analysis of single plaques revealed that instead of just one predominant structure per patient, clouds of overlapping Aβ polymorphisms were present in the brain parenchyma. Upon transmission into transgenic mouse models, the structural variability among patient subtypes could be transmitted. The results of this study reveal important insight into the heterogeneity of senile plaques morphologies within postmortem end stage AD brain.

Due to the possibility that plaque morphologies change over the course of the disease, the second study investigated the nature and durability of the initial Aβ seeds in transgenic mouse brain. Therefore, two well-characterized transgenic mouse models, APP23 and APPPS1, were used that differ in terms of plaque morphology as well as the respective Aβ40/Aβ42 ratio. Previous studies suggest that the Aβ40/Aβ42 ratio may be decisive for differences in plaque morphology, both *in vitro* and *in vivo*. To study the resistance to *in vivo* degradation of fibril polymorphisms, brain homogenate from aged APP23 and APPPS1 transgenic mice was injected into APP-knockout mice. Aβ cannot be propagated in these mice and the protein is merely broken down in the host environment. Strikingly, in the case of both injected brain homogenates, the Aβ42 isoform displayed an increased resistance to degradation for up to 180 days post injection. Upon reinjection into susceptible mice, similar plaque morphologies to the donor mouse strains could be propagated. This study emphasizes the importance of the Aβ42 peptide in the pathophysiology of AD and displays its ability to propagate strain-like morphologies despite a specific Aβ40/Aβ42 ratio.

The propagation of amyloid deposits within the brain plays an important role in the course of AD. Unfortunately, it is very difficult to follow seeding mechanisms of the Aβ peptide *in vivo* due to the lack of differentiation between initial seeds and the following aggregation process per se. Human Aβ injected intracranially into wild-type mice does not cause plaque deposition over extended incubation periods. In the last study, the three amino acid difference between human and murine Aβ was exploited to distinguish between injected seed and the endogenous Aβ of the murine host. Therefore, three new mouse models of either entirely human or murine origin were characterized and their ability to study the propagation of Aβ seeds within axonally connected areas was evaluated. The mouse strains APP_{swe}-GFR and APP_{swe}-GFR x PS1 G384A both express murine Aβ under the Thy1 promotor with a three-fold overexpression. APP_{swe}-GFR mice did not display any plaque deposition within their normal lifespan, whereas the APP_{swe}-GFR x PS1 G384A mouse strain showed the aggregation of diffuse Aβ deposits from 8 months on, most likely caused by the increase in Aβ42 concentration due to the *PS1 G384A* mutation. A mouse strain comprising entirely human Aβ was generated by crossing the well-characterized APP23 mouse model to the APP-knockout strain. Intracranial injections of brain homogenate from APP_{swe}-GFR x PS1 G384A and APP23 x APP-knockout into non-depositing

APP_{swe}- GFR caused in both cases plaque deposition twelve months post injection. These results demonstrate that cross-seeding between human and murine Aβ is possible. The induction of plaque deposition in a mouse model that does not endogenously deposit over time is a strong proof for the prion-like propagation of the Aβ protein. The observed deposition in the APP_{swe}-GFR mouse model additionally propagated to axonally connected areas like the entorhinal cortex. These newly characterized mouse models now provide a tool to follow the focally injected human Aβ seed during its propagation to axonally connected areas in the endogenous murine Aβ environment. Murine and human Aβ isoforms can be distinguished by commercially available antibodies and ELISAs. The results may give important insights into the *in vivo* propagation of the Aβ protein both in rodents and humans.

The research described in this dissertation provides significant contribution to the discovery of Aβ structural polymorphs in AD patients as well as the implication of the Aβ42 isoform in prion-like propagation and the resistance of distinct plaque morphologies in transgenic mice. Furthermore, the development of a new tool to study seed propagation *in vivo* using newly developed mouse models, will provide further insight into the prion-like seeding of the Aβ protein and may eventually aid in the development of new therapeutics.

2. Synopsis

2.1 Alzheimer's disease

2.1.1 Demographics and pathophysiology

Cognitive impairment at an advanced age is often caused by the pathological lesions that define the neurodegenerative process of Alzheimer's disease (AD). The disease is named after Alois Alzheimer, a clinical psychiatrist and neuroanatomist, whose 1906 presentation at the 37th Meeting of South-West German Psychiatrists in Tübingen reported on *"A peculiar severe disease process of the cerebral cortex"* (Alzheimer 1907). In his report, he described the now world-renowned case of Auguste Deter, a 51-year old female patient hospitalized at the Frankfurt Psychiatric Hospital. Alzheimer followed the patient's case from her admission to the clinic due to paranoia, sleep and memory disturbance, aggression and confusion until her death 5 years later. During autopsy, Alzheimer observed two distinctive neuropathological alterations which he called "miliary bodies" and "nerve cells whose interiors were choked by dense bundles of fibrils". In his presentation, Alzheimer correlated these pathological features with the patient's clinical symptoms – an uncommon approach for that time and the case of Auguste D. was believed to be an unusual cause of age-related dementia. It was not until the 1960s that a large autopsy study of brains taken from demented patients revealed to be consistent with the disease described by Alzheimer in 1906 (Blessed 1968; Katzman 1976; Tomlinson et al. 1968, 1970). Although receiving very little attention for his work, Alzheimer's lecture marked the beginning on research into AD which is nowadays recognized as the most common form of dementia worldwide.

Currently, it is estimated that dementia afflicts more than 50 million people worldwide and AD accounts for 60 – 80% of these cases. According to the World Alzheimer Report 2019, it is estimated that by 2050 patient numbers will increase by a threefold accounting to an annual socioeconomic burden of more than $2 trillion (USD) (Brookmeyer 2007; DeTure & Dickson 2019; World Alzheimer Report 2019). Ageing is the highest risk factor for developing AD - with a prevalence estimated at 10% for individuals over 65 years and almost 40% for those over 80 years (Alzheimer's Association Report 2014; Evans et al. 1989; McKhann et al. 1984). With a constantly increasing longevity of the world population, AD is already approaching epidemic proportions. Since there is no cure or preventative therapy, ongoing research is essential.

Macroscopically, the brain of a patient diagnosed with AD shows cerebral cortical atrophy primarily involving the frontotemporal association cortex but often omitting primary motor, sensory and visual areas (Braak et al. 1993). Additionally, a significant atrophy of the hippocampus with an associated dilatation of the lateral ventricles is visible (Perl 2010). During the course of the disease, brain atrophy can become increasingly pronounced involving other brain areas. However, atrophy is not specific to AD and can be seen in other age-related disorders as well as physiological ageing (Alzheimer's Association Report 2014; DeTure & Dickson 2019; Fox & Schott 2004). Clinical AD cannot be

definitively diagnosed until post-mortem neuropathological evaluation. As first observed over 100 years ago, there are two pathological hallmarks that characterize the disease: Extracellular deposits and neurofibrillary tangles or "miliary foci" and "neurofibrils" as first described by Alois Alzheimer.

Extracellular deposits in the brain parenchyma consist of aggregated forms of the amyloid-beta (Aβ) protein (Alzheimer 1907, 1911; Glenner & Wong 1984a). The Aβ protein exists in various sizes containing 37 to 49 amino acid residues but predominantly comprises a length of either 40 or 42 amino acids ($A\beta_{40}$ and $A\beta_{42}$) that are cleaved from the Amyloid Precursor Protein (APP). Apart from its deposition in the brain parenchyma, the Aβ peptide deposits in cerebral blood vessels, called cerebral amyloid angiopathy (CAA). An estimated amount of 85-95% of all AD cases have at least in part some CAA (DeTure & Dickson 2019). Interestingly, amyloid deposits in CAA are enriched in the 40 amino acid long form of the Aβ protein, while parenchymal deposits are enriched in the 42 amino acid long species (Perl 2010; Serrano-Pozo et al. 2011).

Intracellular neurofibrillary tangles are composed of filamentous tau proteins, a splicing product of the microtubule-associated protein tau (MAPT) (Grundke-Iqbal et al. 1986). Under physiological conditions, tau interacts with a range of different proteins and thereby serves important scaffolding functions. Most importantly, it stabilizes the protein tubulin to assemble microtubules and regulates motor-driven axonal transport (Brandt & Leschik 2005). Six tau isoforms exist in the adult human brain tissue including isoforms with 3-repeats and 4-repeats in the microtubule binding domain. Tau proteins in AD are hyperphosphorylated and abnormally folded and therefore lose their physiological roles in binding and stabilizing microtubules in the axon (Alonso et al. 1994, 2018). Neurofibrillary tangles in AD include all 6 isoforms of the tau protein.

Additionally, AD brains are characterized by an inflammatory response mediated by microglia and astrocytes. Activated microglia can be observed in AD tissue frequently surrounding Aβ aggregates and their processes often interfere with the plaque periphery. Reactive astrocytes are also observed around deposits, however in general in lower abundance and in a greater distance to the plaques compared to microglia (McGeer et al. 1987; Shao 1997; Zotova et al. 2011). Whether the glial-mediated inflammatory response observed in AD tissue is a consequence or a cause of neurodegeneration is still a subject of current research.

Clinically, typical AD starts with deficits in short-term memory, word-finding, and language difficulties and gradually progresses to global cognitive impairment. The cognitive deficits can be accompanied by a variety of abnormal neurological and psychiatric symptoms that increase in frequency and severity as the disease progresses (DeTure & Dickson 2019). However, AD can also present with varying phenotypes, severities of the condition and progression rates (Devi & Scheltens 2018; Ferreira 2018;

Ferreira et al. 2017; Friedland et al. 1988). Hence, the purely clinical diagnostic criteria for AD have a poor accuracy and display a sensitivity and specificity of around 70-80% when related to neuropathology (Beach et al. 2012; Knopman 2001). For these reasons, new diagnostic tools and biomarkers to support the clinical diagnosis of AD are needed. A correct diagnosis of AD is currently important to initiate early treatment with acetylcholine esterase (AChE) inhibitors and NMDA-receptor antagonists (Blennow et al. 2006), which are both symptomatic drugs with limited beneficial effects on cognitive symptoms. Efficient clinical biomarkers will be even more important as soon as disease-modifying drugs, such as secretase inhibitors or Aβ immunotherapies, will be available.

Current AD biomarkers include immunoassays for Aβ and tau in cerebrospinal fluid (CSF). While $A\beta_{40}$ or „total" Aβ do not show any differences between AD patients and controls, $A\beta_{42}$ shows a distinct reduction in CSF samples from patients and has already been validated in numerous papers (Motter et al. 1995; Olsson et al. 2016) Interestingly, the changes in CSF $A\beta_{42}$ content occur before the onset of first cognitive symptoms suggesting the presence of a long preclinical phase of the disease with pathology beginning a decade or longer before the onset of first cognitive symptoms (Bateman et al. 2012a; Dubois et al. 2016) The introduction of radioactive positron emission tomography (PET) ligands, like Pittsburgh compound B (PiB), that bind fibrillar Aβ deposits *in vivo* further enables the analysis of the progressive protein deposition in AD brains (Klunk et al. 2004).

2.1.2 APP, Aβ and the amyloid cascade hypothesis

In 1984, Glenner and Wong reported the amino acid sequence of the Aβ peptide – a 4 kiloDalton (kDa) protein isolated from cerebrovascular amyloid derived from patients with Down Syndrome (Glenner & Wong 1984b). One year later, the same amino acid sequence was isolated from senile plaques from AD and Down Syndrome patients (Masters et al. 1985). Subsequently, four different groups in 1986 and 1987 were able to isolate the gene encoding the amyloid precursor protein (APP) mapped on chromosome 21 and giving rise to the Aβ peptide (Gorevic et al. 1986; Kang et al. 1987; Roher et al. 1986; Selkoe et al. 1986).

The APP protein belongs to a gene family that additionally encodes the Amyloid precursor-like proteins 1 and 2 (APLP1 and APLP2). APLPs do not give rise to the Aβ peptide but share a similar structural organization with partially overlapping functions (Slunt et al. 1994; Wasco et al. 1992, 1993). The APP protein is a type I single-pass transmembrane protein with a large extracellular ectodomain and a short cytoplasmic part. Alternative splicing of the APP protein leads to 3 different major isoforms: the APP_{695} amino acid form which is predominantly expressed in neuronal tissue with a particularly strong expression in the cortex and the hippocampus but lack of expression in glia cells, as well as the APP_{751} and APP_{770} isoforms that are expressed in peripheral organs and fibroblasts(Guo et al. 2012; Hick et al. 2015; Wang et al. 2005).

Interestingly, the precise physiological function of APP is not known and remains subject of investigation. The APP protein has no enzymatic activity and signal transduction therefore relies on interaction with other proteins. To date, more than 200 extracellular and intracellular binding partners have been identified although only a few of them have been verified *in vivo* (Deyts et al. 2016; Perreau et al. 2010). Many studies point towards a positive effect of APP expression on cell health and growth. One study using transgenic mice overexpressing wildtype APP showed enlarged neurons (Oh et al. 2009). In *in vitro* transfected cell lines, APP favors cell growth, motility, neurite outgrowth and cell survival. In adult animals, intracerebral injections of the APP ectodomain can improve cognitive function and synaptic density (Meziane et al. 1998; Roch 1994).

Deletion of APP in mature mice (and thus Aβ production) produces only little phenotype and does not suggest that a loss of APP or Aβ function is deleterious to the adult animal. $APP^{-/-}$ mice have been extensively studied and show a mild reduction in brain and body weight, reduced grip strength, increased susceptibility to injury and age-dependent deficits in neuronal morphology, synaptic plasticity and behavior (Li et al. 1996; Müller & Zheng 2012; Ring et al. 2007; Steinbach et al. 1998; Zheng et al. 1995). Triple knockouts involving APP, APLP1 and APLP2 as well as $APP^{-/-}$, $APLP2^{-/-}$ mice and $APLP1^{-/-}$, $APLP2^{-/-}$ mice die within a short time after birth due to severe neuromuscular deficits(Herms et al. 2004). In contrast, knockout mice of APP and APLP1 are viable which indicates that APLP2 has unique properties that are required when either APP or APLP1 is absent. This suggests that APP family

members have overlapping functions when being co-expressed and might be an explanation for the minor phenotypes observed in the single knockouts.

APP processing

The APP protein is quickly metabolized and a variety of pathways exist for proteolysis that result in many different biologically active fragments each having specific or even opposing functions. APP is sorted in the endoplasmic reticulum and Golgi network and afterwards delivered to dendritic or axonal membrane compartments by fast axonal transport (Koo *et al*. 1990). The following steps in APP processing depend on the cellular distribution of the protein. From the Golgi network, APP can be transported to the cell surface or to an endosomal compartment. The surface accumulation of the APP protein favors subsequent non-amyloidogenic processing whereas accumulation in endosomal compartments promotes amyloidogenic processing (Greenfield et al. 1999; Haass & Selkoe 1993; Haass et al. 1993; Hartmann et al. 1997; Sisodia 1992; Xu et al. 1997).

The non-amyloidogenic pathway is physiologically predominant, can be stimulated by neuronal and synaptic activity and is performed by sequential cleavage of the α- and γ-secretases (Roberts et al. 1994). ADAM10 (disintegrin and metalloproteinase domain-containing protein 10) is the major α-secretase in the brain that occurs on the cell surface (Kuhn et al. 2010). The α-secretase cleaves APP within the Aβ region liberating the large soluble ectodomain sAPPα and thereby preventing the formation of Aβ (Esch et al. 1990; Sisodia et al. 1990). The residual membrane-bound C-terminal fragment (α-CTF) is then cleaved by the γ-secretase liberating both the p3 peptide and the intracellular C-terminal fragment (AICD). In contrast to the Aβ peptide, studies suggest that the sAPPα fragment has a neuroprotective function in neuronal plasticity and survival (Furukawa et al. 2002; Mattson 1997). The p3 fragment has no currently known physiological role and the AICD domain may have a function in nuclear signaling (Cao & Sudhof 2001; Gu et al. 2001; Sastre et al. 2001; Weidemann et al. 2002).

The Aβ peptide is located within the ectodomain and continues into the transmembrane region of the APP protein. The amyloidogenic pathway that generates Aβ is initiated within endosomes where both the β-secretase 1 (BACE1), a transmembrane aspartic protease, as well as the γ-secretase are located (Fukumori et al. 2006; Kinoshita et al. 2003; Parvathy et al. 1999). BACE1 is able to cleave APP at two different sides within the Aβ protein: the +1 (prior to amino acid 1) or +11 site thereby liberating sAPPβ (Cai et al. 2001). Subsequent to BACE1 cleavage and release of the sAPPβ ectodomain, the residual APP C-terminal fragment is cleaved by the γ-secretase complex varying from +40 to +44 to generate the Aβ peptide and the AICD domain (Haass 2004). The Aβ protein is then dumbed from the cell into the extracellular space. In addition to its pathophysiological function in the onset of AD, the Aβ peptide plays a physiological role in synaptic function, regulating synaptic scaling (Kamenetz et al. 2003)and synaptic vesicle release (Abramov et al. 2009).

Recently, a new processing pathway for APP has been characterized that cuts N-terminally of the β-secretase cleavage site. The so called η-cleavage pathway produces fragments about 92 or 108 amino acids long that end at either the β- or α-secretase site. The Aη-α peptide is a potentially synaptotoxic fragment since it has been shown to suppress synaptic plasticity *in vitro* as well as neuronal activity in mouse brain (Willem et al. 2015).

γ-secretase activity

The γ-secretase is a multiprotein complex whose proteolytic activity cleaves APP within the transmembrane (TM) domain (Haass & Selkoe 1993). Due to its high complexity, more than a decade was required to define its components and way of action. Since then, the γ-secretase has been described as a multiprotein complex composed of either presenilin 1 (PS1) or presenilin 2 (PS2) and three additional co-factor proteins: Nicastrin (Nct), which is a type I transmembrane glycoprotein, and anterior pharynx 1 (Aph-1) and PSEN enhancer 2 (Pen-2) which are two multipass transmembrane proteins (Bergmans & de Strooper 2010; de Strooper 2003; Iwatsubo 2004; Kimberly et al. 2003).

The γ-secretase processes the TM domain of APP into Aβ peptides, thereby mostly producing the benign $A\beta_{40}$ species as well as lesser amounts of the longer, more aggregation prone and pathogenic species $A\beta_{42}$. Apart from the two major Aβ cleavage products, mass spectrometry studies could show that γ-secretase cleavage additionally gives rise to Aβ species of varying lengths from $A\beta_{38}$ to $A\beta_{49}$ (Matsumura et al. 2014). APP is processed via two major pathways: γ-secretase starts endoproteolysis at the ε-cleavage which starts after +49 or +48 generating $A\beta_{49}$ and $A\beta_{48}$ and two different AICDs, respectively (Kakuda et al. 2006; Sato et al. 2003). The cleavage products $A\beta_{49}$ and $A\beta_{48}$ are then sequentially cleaved in steps of three amino acids to produce $A\beta_{40}$ and $A\beta_{42}$ (Takami et al. 2009). The cleavage steps are therefore: $A\beta_{49} \rightarrow A\beta_{46} \rightarrow A\beta_{43} \rightarrow A\beta_{40}$ and $A\beta_{48} \rightarrow A\beta_{45} \rightarrow A\beta_{42}$ (Fernandez et al. 2014). Another peptide, $A\beta_{38}$, can be formed from both pathways, being cleaved from $A\beta_{42}$ and $A\beta_{43}$ (Okochi et al. 2013).

PSs are the crucial catalytic component of the γ-secretase and comprise nine TM domains (Kimberly et al. 2003; Li et al. 2000). Nascently produced PS is inactive. To gain functionality, it requires endoproteolytic cleavage between TM6 and TM7 to generate a 27–28 kDa amino-terminal fragment (NTF) and a 16–17 kDa carboxyl-terminal fragment (CTF). The formation of a mature γ-secretase complex starts with an initial scaffolding complex composed of Aph-1 and nicastrin (LaVoie & Selkoe 2003). The Aph-1-nicastrin subcomplex is then accompanied by the PS holoprotein that binds with its proximal C-terminus to the TM domain of nicastrin (Jiang et al. 2014; Kaether et al. 2004). After PS binding, Pen-2 is incorporated by interacting with one of the nine TM domains of PS (Kim et al. 2005; Watanabe et al. 2005). Finally, the loop domain between TM6 and TM7 of PS1 is cleaved by endoproteolysis to activate the catalytic function of PS as described above (Fukumori et al. 2010). In

2015, the first cryoEM structure of the y-secretase complex was published (Bai et al. 2015) and recently the same lab presented a high-resolution cryo-electron microscopy structure of γ-secretase interacting with a fragment of APP. The structure illustrates that upon binding, the C-terminal α-helical turn of the fragment unwinds thereby exposing its peptide bonds and the distal parts of the C-terminus participate in a β-sheet structure formed with the PS protein (Zhou et al. 2019).

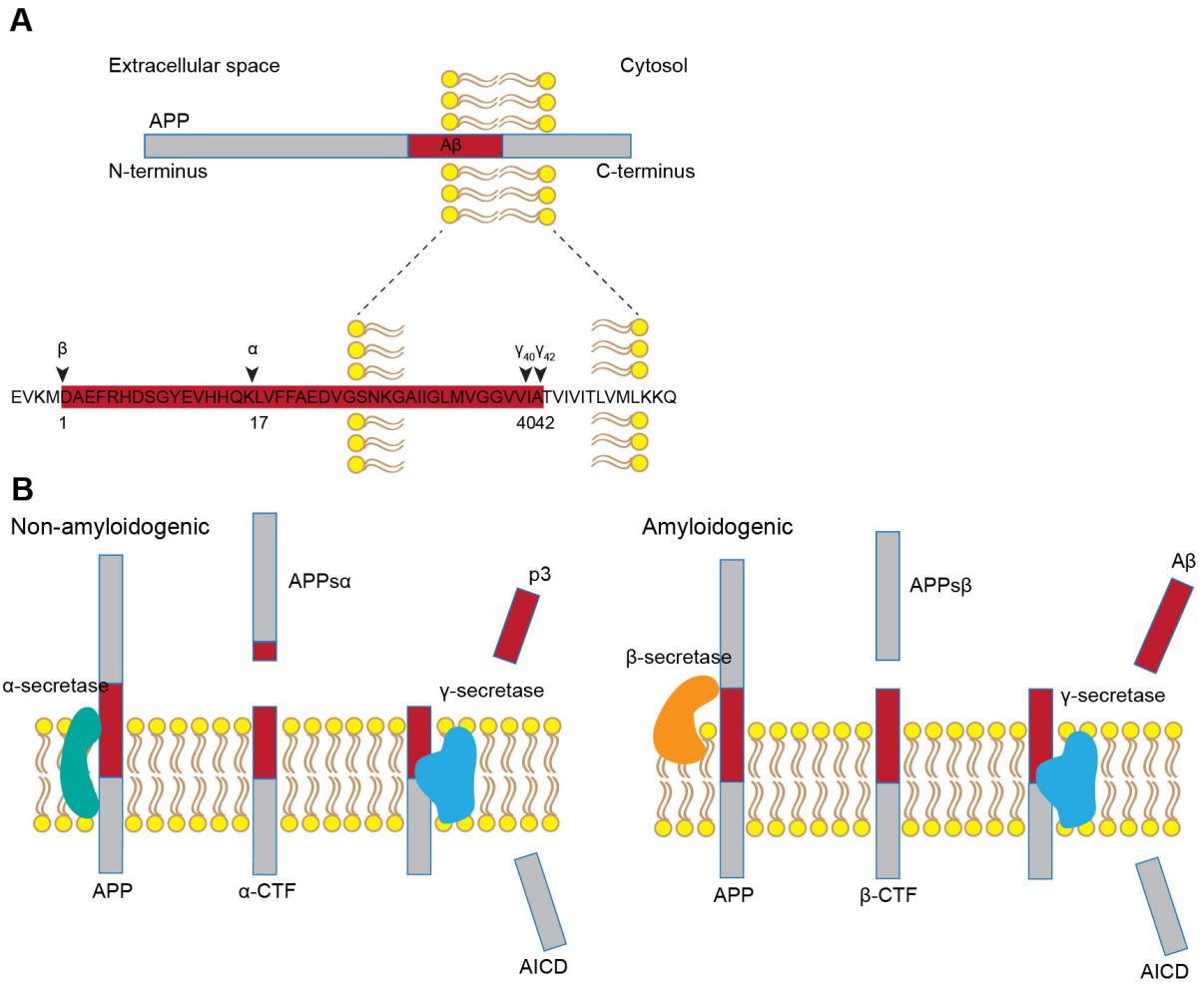

Figure 1. Cleavage of the Amyloid Precursor Protein (APP). (A) The APP protein is a transmembrane protein with a large N-terminal ectodomain and a shorter C-terminus. The Aβ peptide comprises a lengths of generelly 40 or 42 amino acids and starts within the ectodomain comprising into the transmebrane region (depicted in red). **(B)** Non-amyloidogenic processing involves the sequential cleavage of the α-secretase followed by cleavage of the γ-secretase. Created products are APPsα, α-CTF and p3. Amyloidogenic processing involves the sequential cleavage of β-secretase and γ-secretase. The resulting products are β-CTF, APPsβ and Aβ.

Genetics of Alzheimer's disease

AD is an age-related disorder and in accordance therewith, most cases of AD present as late-onset AD (LOAD) which is defined as AD with an age at onset later than 65 years of age. On the contrary, early-onset AD (EOAD) represents the minority of diseases with only 1% to 6% of all cases and age of onset varies roughly between 30 and 65 years of age. On a genetical background, AD can be further divided into familiar AD (FAD) with Mendelian inheritance as well as sporadic AD (SAD) without a positive family history. While FAD cases mostly manifest as EOAD, rare LOAD cases have been described, as well (Bekris et al. 2010). In general, more than 95% of all AD cases appear to be sporadic, usually with an old age at onset.

With an estimated prevalence of less than 5%, autosomal dominant cases of FAD are very rare. However, the clinicopathological characteristics of FAD are indistinguishable from the more common sporadic forms of the disease (Shepherd et al. 2009). Hence, great efforts have been made studying the FAD linked genes and thereby significant progress has been made in revealing the mechanisms underlying AD pathogenesis. Mutations in the 3 genes encoding for APP, PS1 and PS2 are causative in the majority of FAD cases. To date, there are around 32 APP, 180 PSEN1 and 14 PSEN2 gene mutations that result in EOAD (Goate 2006; Haass & Strooper 1999; Levy-Lahad et al. 1995a; Sherrington et al. 1995). More recently, an additional copy of the APP gene has been identified as the cause of illness in families with a history of AD (Sleegers et al. 2006; Thonberg et al. 2011).

Mutations in the APP gene can be categorized into three classes: close to the BACE cleavage site, close to the γ-secretase cleavage site and within the mid-domain region of the Aβ peptide. One of the first APP mutations described, APPV717I (London), is located within the γ-secretase cleavage domain (Goate 2006). The effects of this mutation have been extensively studied and result in an increase in $A\beta_{42}$ levels without a noticeable effect on $A\beta_{40}$ levels. In general, almost all mutations around the γ-secretase cleavage site change the cleavage position of Aβ, such that the ratio of $A\beta_{42}/A\beta_{40}$ is tipped in favor of $A\beta_{42}$. Mutations in the mid-domain of Aβ can have various effects that are currently not well understood: A variety of different mutations at codon E693 have been described, among them Arctic mutation E693G, Dutch mutation E693Q, Italian mutation E693K, all leading to different phenotypes such as AD, vascular dementia or mixed phenotypes (Bugiani et al. 2010; Kalimo et al. 2013; Maat-schieman et al. 2005; Miravalle et al. 2000). Another well-studied APP mutation in Swedish familiar Alzheimer's disease causes a two amino acids substitution close to the BACE cleavage site: K670N and M671L (Swedish). These mutations increase the rate of APP proteolysis resulting in an increased supply of the APP fragment sAPPβ to be sequentially cleaved by γ-secretase to produce all Aβ species (Mullan et al. 1992; Nilsberth et al. 2001; Scheuner et al. 1996).

Of note, the APP gene is mapped to chromosome 21, which explains the observation that most patients with Down syndrome, or trisomy 21, develop neuropathological features of AD when they reach their

40s (Lott & Head 2019). For a long time, it was considered that all APP mutations are autosomal dominant and cause the disease with complete penetrance. However, one case of a recessive inheritance concerning the mutation A673V and corresponding to position 2 of Aβ was described in 2009 with heterozygous carrier of the mutation being totally unaffected (di Fede et al. 2009). It is important to note that a different amino acid substitution at exactly the same site, A673T, protects against the protein accumulation and therefore against AD (Jonsson et al. 2012).

Mutations in the genes encoding for *PS1* and *PS2* cause the majority of familiar forms of AD. *PS1* is located on chromosome 14 (locus14q24.3) and *PS2* on chromosome 1 (locus 1q31-q42) and both genes share a high homology. There are more than 180 FAD-linked *PS1* mutations, whereas mutations in *PS2* are a much rarer cause of FAD since PS2-containing γ-secretase complexes do not have a major role in mediating Aβ production (Herreman et al. 1999). In general, mutations in both *PS1* and *PS2* cause a partial loss of protein function. In wild-type *PS*, the $A\beta_{40}$ variant represents the major peptide product. γ-secretase cleavage starts at residues +49 or +48 (ε-cleavage) and then progresses in a stepwise fashion to produce the shorter forms of Aβ as described above (Takami et al. 2009; Yagishita et al. 2006). After each tripeptide cleavage, a proportion of the cleaved peptide diffuses away from the γ-secretase. In case of a reduced efficiency of the γ-secretase complex due to mutations, the time frame for longer forms of the peptide to diffuse away between the single cleavage events increases. This assumption likely explains both the reduction in the total amount of Aβ as well as the simultaneous increase in the proportion of longer forms of Aβ resulting in an increased $A\beta_{42}/A\beta_{40}$ ratio that are frequently observed in PS mutations (de Strooper 2007; Karran et al. 2011; Wolfe 2007).

In conclusion, it can be summarized that for the FAD-linked *APP* and *PS* mutations two potential effects that predispose individuals to EOAD exist: an increase in the amount of $A\beta_{42}$ or an increase in the $A\beta_{42}/A\beta_{40}$ ratio that is sufficient even in the context of an overall reduction in Aβ production (Hellstrom-Lindahl et al. 2009).

Sporadic AD

Whereas increased Aβ production is the cause for familiar AD, sporadic forms of the disease are rather induced by disturbance in Aβ clearance mechanisms (Bateman et al. 2006; Mawuenyega et al. 2010). Sporadic AD is considered to be multifactorial, however it is accompanied by a strong genetic predisposition with a heritability estimate of 60-80% (Gatz et al. 2006). The genetic component itself is extremely complex and gene mutations or polymorphisms may interact with each other. In addition, environmental factors like high cholesterol, hypertension, atherosclerosis, coronary heart disease, diabetes and obesity increase the risk of sporadic disease (Alford et al. 2018; Carnevale et al. 2016; Ott et al. 1999).

Among the first genes discovered by genome wide association studies that increase the risk of developing AD was Apolipoprotein E (ApoE), a polymorphic glycoprotein expressed in liver, brain, macrophages and monocytes (Nalbantoglu et al. 1994; Poirier et al. 2014). The protein is secreted by glial cells and involved in transport of cholesterol and other lipids within the brain (Rigat et al. 1990). It exists in three allelic variants, ApoEε2, ApoEε3, and ApoEε4. Only ε4 increases AD risk in a dose-dependent manner and ε2 decreases disease risk whereas ε3 can be considered neutral (Corder et al.; Farrer et al. 1997; Roses & Saunders 1994). According to epidemiological studies, carrying just one ApoEε4 allele increases AD risk by 3-4 fold and carrying two ApoEε4 alleles increases the risk 9-15 fold (Genin et al. 2011; Neu et al. 2017). One of the major routes by which Aβ is cleared from the brain involves the vasculature (Ueno et al. 2014). It has been shown that ApoE binds to Aβ and mediates the clearance of the protein from the brain into the periphery with ApoEε2, ApoEε3, and ApoEε4 being increasingly less effective at clearance (Deane et al. 2008). This assumption has recently been supported by the first functional 3-dimensional model of CAA in bioengineered human vessels (Robert et al. 2017).

Large-scale GWAs studies have identified additional new risk factors that mainly fall into three different groups: cholesterol and lipid metabolism, immune system and inflammatory response as well endosomal vesicle cycling. One of these risk factors, Triggering Receptor Expressed On Myeloid Cells 2 (TREM2) has recently gained a lot of attention (Guerreiro et al. 2013; Jonsson et al. 2013). A rare missense variant in TREM2, R47H, increases risk for AD roughly comparable to the effect size of the ApoEε4 allele. TREM2 is exclusively expressed by microglia and enhances their phagocytic activity (Jiang et al. 2013; Neumann & Takahashi 2007; Sessa et al. 2004). Microglia are activated in AD and many studies report a decreased microglial activation and plaque-association in mice deficient for TREM2 (Jay et al. 2015; Ulrich et al. 2014; Wang et al. 2016). This inability to cluster around plaques is associated with defects in plaque compaction, microglia proliferation and high levels of dystrophic neurites (Wang et al. 2015; Yuan et al. 2016).

The risk factors shortly mentioned here underline the importance of physiological Aβ clearance from the brain to the periphery and their implications for the onset of AD. Aβ peptides can be removed from the brain by multiple clearance mechanisms including enzymatic degradation and cellular uptake, blood-brain barrier transport, interstitial fluid bulk flow facilitated by astroglial channels and CSF routes into the blood stream and lymphatic system (Tarasoff-Conway et al. 2015). Changes in any of the systems listed may contribute to the accumulation of Aβ and result in the ultimate deposition of aggregates.

The amyloid-cascade hypothesis – a concept to explain the origin of AD

During the past decades, many hypotheses regarding the pathogenic causes for AD have been put forward. Among these, the amyloid cascade hypothesis is by far the most influential one and drastically changed AD research since its first introduction in 1992 (Hardy & Allsop 1991).

The original amyloid cascade hypothesis postulates that the observed neurodegeneration and subsequent dementia in AD is directly triggered by abnormal accumulation of the Aβ protein within the brain parenchyma. The histopathologically visible Aβ deposits are considered the direct cause of interference with synaptic function thereby causing severe and irreparable changes. With time, inflammatory responses in form of microgliosis and astrocytosis in the vicinity of fibrillar deposits can be observed that further enhance neuronal injury. The events eventually end up in oxidative stress as well as changes in ionic homeostasis. Oligomerization and hyperphosphorylation of the tau protein into neurofibrillary tangles contribute to additional toxic effects. The cascade finally peaks in overall neuronal dysfunction and cell death causing the progressive dementia that characterizes the clinical symptoms of AD (Beyreuther et al. 1991; Hardy & Allsop 1991; John A. Hardy & Gerald A. Higgins 1992; Selkoe 1991).

Originally, two key observations resulted in the formulation of the amyloid cascade hypothesis: The verification that Aβ is the key component of the extracellular deposits in AD (Glenner & Wong 1984b) as well as mutations in APP (Goate et al. 1991), PS1 and PS2 (Levy-Lahad et al. 1995b; Sherrington et al. 1995) which cause familiar forms of the disease and are directly linked to the presence of Aβ. Since familiar forms and sporadic forms of the disease have similar clinical phenotypes, the amyloid cascade hypothesis is used as a general explanation for all types of AD (Reitz 2012). Further support for Aβ being the initial trigger of the disease comes from humans suffering from trisomy 21 that have three copies of the APP gene and inexorably develop neuropathologically typical AD. Trisomy 21 patients that die at young age due to other causes only present with Aβ plaques without neurofibrillary tangles and neuritic dystrophy thereby placing the protein deposition at the first position of the cascade (Prasher et al. 2010; Rovelet-Lecrux et al. 2006). Regarding the timely sequence of Aβ deposits and neurofibrillary tangles, clinical and biomarker changes in familial forms of the disease suggest that first Aβ deposition occurs followed by tau hyperphosphorylation (Bateman et al. 2012a; Lemere et al. 1996b,a). Furthermore, familiar mutations within the tau gene cause frontotemporal lobe dementia, a specific form of dementia that is clinically characterized by neurofibrillary tangles but without Aβ deposition. Mutations within the tau gene do not cause familiar forms of AD although tau pathology alone is sufficient to cause progressive neurodegeneration (D'Souza et al. 1999; Goedert & Spillantini 2000; Hutton et al. 1998; Polanco et al. 2018).

Recently, considerable concern regarding the validity of the amyloid cascade hypothesis has been raised and therefore, some modifications of the original hypothesis have been made. It has been shown in many studies, that the deposition of Aβ does neither correlate with the presence of neurofibrillary tangles, nor

cell loss or dementia (Delacourte et al. 1999; Gomez-Isla et al. 1997; Savva et al. 2009). One hypothesis incorporating these data posits that rather small soluble oligomers than fibrillar deposits of Aβ represent the neurotoxic agents causing synaptic damage in the disease (Walsh & Selkoe 2007). Especially the relative increase of $A\beta_{42}$, which is the more aggregation prone form of Aβ and increased in familiar forms of AD, seems to enhance oligomer conformation. As a consequence, oligomeric Aβ is able to act at a distance from plaques mediating neuronal loss (Ferreira & Klein 2011; Haass & Selkoe 2007; Hayden & Teplow 2013; Hefti et al. 2013; Koffie et al.; Mucke & Selkoe 2012; Viola & Klein 2015). Another hypothesis claims that a certain level of Aβ deposition triggers "aggregate stress" that sufficiently causes tau pathology which in turn leads to neuronal loss independent of Aβ (Karran et al. 2011). As all known FAD mutations increase the probability of Aβ deposition, it could very well be the case that Aβ is the first trigger of the disease, however the subsequent neurodegeneration is driven by a complicated process of cellular actions leading to irreversible disturbances of normal brain homeostasis (Strooper & Karran 2016). The fact that there is a long clinically silent phase between amyloid deposition and first signs of dementia generally supports this assumption.

Apart from the amyloid cascade hypothesis, there are a few alternative explanations for the onset and progression of AD: The mitochondrial cascade hypothesis that claims that mitochondrial dysfunction during aging leads to the onset of disease (Swerdlow & Khan 2004; Swerdlow et al. 2014), the metabolism hypothesis positing that the underlying cause of AD is cerebral glucose hypometabolism (Hoyer 1988, 1991). The vascular hypothesis is based on neuropathological observations of a reduced vascular network in the AD brain and may provide important insights for sporadic forms of the disease. Interestingly, hypertension and diabetes which display significant vascular morbidities, are risk factors for AD and studies using electron microscopy could show that fibrillary Aβ first appears in the perivascular space. This work relates to an impairment of Aβ clearance from the brain, like the glymphatic system, as suggested for SAD (de la Torre 1994; Fischer et al. 1990; Iliff et al. 2012; Kumar-Singh et al. 2006; Marchesi 2011; Welander et al. 2009).

The various hypothesis presented within this chapter may likely all play a role in the pathophysiology of AD. However, it is important to distinguish between the causes and the consequences of the disease and the amyloid cascade hypothesis represents currently the best and most conclusive model to explain the onset of AD (**Figure 2**).

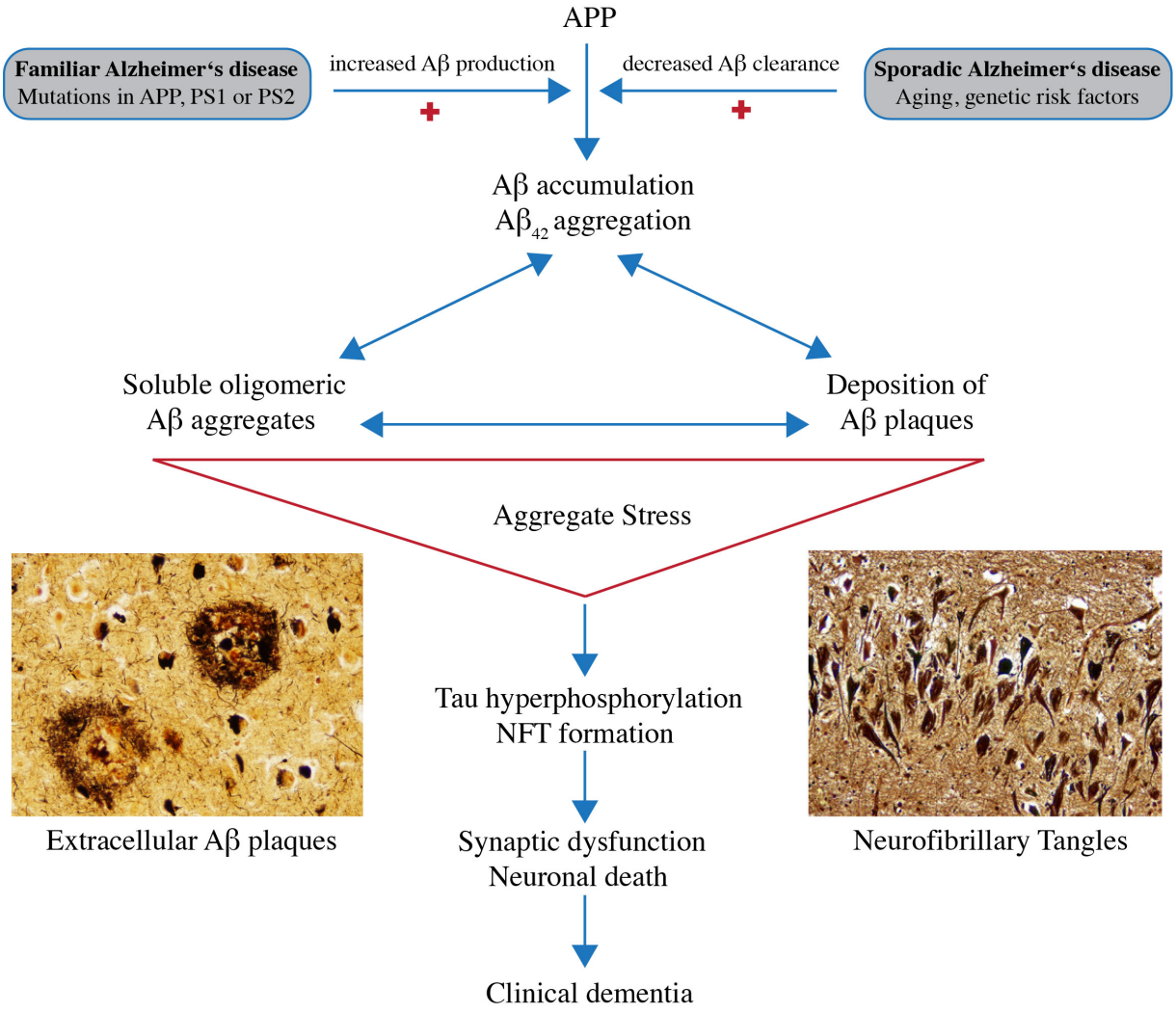

Figure 2. Schematic illustration of the amyloid cascade hypothesis. According to the amyloid cascade hypothesis, the accumulation and subsequent deposition of the Aβ protein is the initial step that ultimately leads to the onset of AD. Familiar forms of the disease comprising mutations in the genes APP, PS1 or PS2 cause an increased Aβ peptide production, whereas sporadic Alzheimer's disease is likely caused by decreased Aβ clearance or degradation. Both scenarios cause an accumulation of the Aβ protein over time resulting in the build-up of both soluble oligomeric Aβ aggregates as well as deposition into senile Aβ plaques. It is not clear, if neurotoxicity is mainly mediated by soluble aggregates or rather deposited plaques. To combine the potential mechanisms of neurotoxicity, the term aggregate stress is used here. Aβ aggregate stress is followed by tau hyperphosphorylation into neurofibrillary tangles, neuronal death and ultimately the onset of clinical symptoms.

2.1.3 Experimental models to study Alzheimer's disease

While less than 5% of AD cases are caused by familial inheritance, these mutations serve as the basis for the majority of animal models that are currently used to mimic AD. This is due to the specificity of most age-related neurodegenerative diseases to humans and missing clinical phenotypes in animal species although some show aspects of brain aging (Gerlach & Riederer 1996; Jucker 2010; Walker & Jucker 2017). Interestingly, mammals like nonhuman primates, bears, dogs and sheep are susceptible for the development of the pathological hallmarks of AD but these characteristics are complete missing in aged laboratory rodents (Price et al. 1991; Rosen et al. 2008; Walker 1997). Due to the lack of natural models, the development of transgenic animal models based on familial AD mutations has been of inestimable worth.

Most of the transgenic mouse models used at present overexpress mutant human APP or PS1 or both combined. These mice develop cerebral Aβ deposits, CAA or a mixture of both and Aβ plaques are often associated with dystrophic neurites, synapse breakdown as well as microgliosis and astrocytosis (Duyckaerts et al. 2008). CAA is often associated with damage of smooth muscle cells, vascular rupture and can even result in microbleeds (Herzig et al. 2006). Models that do not contain familiar mutations but model the overexpression of wild-type human APP have been described as well. These mice develop parenchymal Aβ deposits and vascular deposition at an advanced age (Bodendorf et al. 2002; Herzig et al. 2004). In general, these mouse models mimic the Aβ-associated hallmarks of AD very well. However, unlike in AD brains, neuronal loss is modest and confined to hippocampal and cortical regions and APP and PS transgenic mouse lines do not develop neurofibrillary tangles (Calhoun et al. 1999; Gómez-Isla et al. 1996; West et al. 1994). The latter might be due to the difference in tau splice variants between human beings and mice. As already stated above, tau is expressed in six isoforms in the human brain, with either 3-repeats or 4-repeats in the microtubule binding domain whereas mice only express the four-repeat form. Tau aggregation has been shown to be influenced by its isoforms involved and neurofibrillary tangles in AD tissue consist of all 6 isoforms (Lee et al. 2001). Importantly, rats express all six isoforms of the tau protein. To model tau pathology in AD mouse models, overexpression of human tau protein with mutations causing frontotemporal dementia pathology are used (Lewis et al. 2001; Oddo et al. 2003). Since tau mutations do not have an implication in AD, the pathophysiology in mice induced by these mutations is difficult to judge. Modelling of the Aβ-tau connection therefore provokes difficulties although studies could show that crossing APP transgenic and tau transgenic mice as well as intracerebral injections of $A\beta_{42}$ into tau transgenic mice accelerates tau phosphorylation and NFT pathology (Götz et al. 2001; Lewis et al. 2001). An effect that might in part be regulated by the tau kinase glycogen synthase kinase 3β (GSK3β) (Terwel et al. 2008). Interestingly, a recently characterized transgenic rat model expressing both mutant APP and PS1 does not only develop age-dependent Aβ deposition but also tauopathy rendering it an important model for future AD research (Cohen et al. 2013). Another disadvantage of most transgenic mouse models is the unphysiologically high

overexpression of the mutated APP gene including all its cleavage products as well as the gene insertions at various places within the genome, thereby possibly interfering with other mouse genes. To characterize possible artificial phenotypes of the current overexpression models, Saito *et al*. developed single APP knock-in mouse models harboring different familial mutations in the APP gene thereby overproducing $A\beta_{42}$ without overexpression of APP (Saito et al. 2014).

When studying AD, there are many different rodent models to choose from. Two of the most well-studied transgenic mouse models are the lines APPPS1 and APP23 (Radde et al. 2006; Sturchler-Pierrat et al. 1997) – both of them have been used in this thesis. The APP23 mouse line expresses the human APP_{751} isoform under the thymocyte differentiation antigen 1 (Thy-1) promotor which is exclusively expressed in neurons and without extraneural expression (Andrä et al. 1996; Caroni et al. 1997; Gordon et al. 1987; Vidal et al. 1990). The APP gene harbors the Swedish mutation (KM670/671NL) causing first parenchymal deposits at around 6 months of age in form of large and rather diffuse deposits followed by CAA at around 12 months of age (Radde et al. 2006). In comparison, the APPPS1 mouse model does not only express the Swedish mutation but additionally bears PSEN1 containing an L166P mutation, both under the control of the Thy-1 promotor. Expression of the human APP transgene is around 3 times higher than the endogenous murine APP and $A\beta_{42}$ is produced in increased quantities. The mouse model shows an early-onset of plaque deposition at around 1,5 months of age. Plaques appear small and very compact and almost no CAA is generated (Radde et al. 2006).

It should be emphasized that no currently existing mouse model represents all features of AD. An ideal model would be able to demonstrate both clinical and pathological features of the disease thereby including cognitive and behavioral deficits, Aβ plaques, NFTs, gliosis, synapse loss, axonopathy, neuronal loss and as a consequence broad neurodegeneration. These criteria are reflected differently by various mouse models. While Aβ plaques and cognitive defects are observed in almost all transgenic mice, NFTs are missing if not caused by human tau expression. Neuronal loss is a rare feature that can only be observed in a few disease models. Nevertheless, the tremendous progress in understanding the pathophysiology in AD in recent years were made possible due to the wide range of different mouse models available.

2.1.4 The prion hypothesis, amyloidogenic proteins and neurodegenerative diseases

Prion diseases are neurodegenerative diseases of a fatal spongiform type all caused by an aggregated form of the prion protein, called $PrP^{Scrapie}$ (PrP^{Sc}), that propagates through the brain by forcing its conformation on the physiological form of the prion protein, $PrP^{Cellular}$ (PrP^{C}). In men, prion diseases include kuru (Gajdusek et al. 1966), Creutzfeld-Jakob disease (CJD) (Gibbs et al. 1968), Gerstmann-Sträussler-Scheinker (GSS) syndrome (Masters et al. 1981) and fatal familial insomnia (Medori et al. 1992). In animals, known forms of the disease are scrapie in sheep, goats and mufflons (M'Gowan 1914; Wilson et al. 1950), transmissible mink encephalopathy in mink (Burger & Hartsough 1965), chronic wasting disease in mule, deer and elk (Williams & Young 1980, 1982) and bovine spongiform encephalopathy (BSE) in cows (Bradley & Liberski 2004; Bradley et al. 2006; Collee et al. 2006).

The origin of the prion disorders has been a matter of debate for decades. The first description of a prion disorder, or transmissible spongiform encephalopathy (TSE), dates back to the 18th century when a strange disease affected Merino sheep involving abnormal behavior of "scraping against fences". That kind of behavior was later the reason to call the disease "Scrapie" (Hunter 1997). In 1920, the neurologists Hans Gerhard Creutzfeldt and Alfons Maria Jakob described a human neurological disorder of unknown origins (Jakob 1921) that would eventually change the central dogma of molecular biology: information encoded by nucleic acids can be synthesized, stored and used by an organism to replicate itself (Watson & Crick 1953). The scientific discovery that viral nucleic acids encode genetic information and can be infectious (Fraenkel-Conrat & Williams 1955; Fraenkel-Conrat et al. 1955) directed the first theories regarding the causes of TSEs towards a "slow virus" due to its long incubation times (Eklund et al. 1967; Gajdusek 1967; Sigurdsson & Palsson 1958). In 1959, another human neurological disorder among the Fore tribe in Papua New Guinea, called kuru, was discovered and resembled CJD and scrapie in many ways (Gajdusek & Zigas 1959). In 1967, Alper discovered that the agent causing scrapie could not be inactivated by UV radiation and therefore naturally replicates without nucleic acids (Alper et al. 1967). In the same year, John Griffith was the first scientist to speculate that the toxic agent underlying CJD, kuru and scrapie could be proteinaceous (Griffith 1967). Stanley Prusiner eventually shaped the protein-only hypothesis and coined the term "prion", proteinaceous infectious particle, for which he later won the Nobel Prize. He substantiated his prion hypothesis by isolation of an infectious protein from diseased animals and stopped infectivity by methods that destroy proteins. Of importance, radiation and nucleases that efficiently destroy nucleic acids failed to inactivate the prions (Bolton et al. 1982; Prusiner 1982; Prusiner et al. 1982, 1983).

Human prion diseases manifest in rapidly progressive dementia, myoclonus, visual or cerebellar signs, pyramidal/extrapyramidal signs and akinetic mutism. The clinical symptoms of the disease are preceded by an extremely long incubation phase of several years. However, as soon as the clinical signs become evident, the course of the disease is fast and dramatic(Aguzzi & Calella; Aguzzi et al. 2008).

Histopathological features of human prion diseases typically encompass the triad of spongiform change, neuronal loss and gliosis. The spongiform change is specific to prion diseases and distinguish it from other neurodegenerative diseases that are often accompanied by neuronal loss and gliosis. It is characterized by vacuoles of variable forms in the neuropil of deep cortical layers, the cerebellar cortex or subcortical grey matter and correspond to enlarged neurites containing membrane fragments and amorphous material (Liberski et al. 1992).

Prion diseases are grouped into three classes: familial, sporadic and acquired forms. All forms are characterized by plaques of PrP^{Sc} within the nervous system. Familial prion diseases include genetic CJD, FFI and GSS and are all due to autosomal dominant mutations within the PRNP gene that gives rise to PrP^{C}, the physiological form of the prion protein (Hsiao et al. 1989). Sporadic CJD and sporadic FFI are among the most common forms of disease and currently lack any known etiology (Harries-Jones et al. 1988; Palmer & Collinge 1993). Acquired forms of the disease are caused by infection with previously existing prions. The BSE breakout in the beginning of the 1990s affected almost 280000 cattle and provoked a major food crisis (World Org. Anim. Health. 2007). Additionally, it is believed that transmission of BSE prions in contaminated food led to more than 200 cases of variant CJD (Will et al. 1996) (World Anim. Health. Sit., 2006; Eur. Allied Ctries., 2006). Iatrogenic CJD has been caused by transplantation of corneal or dural tissue from patients unknown to be suffering from TSEs or by neurosurgery due to instruments incompletely sterilized after surgery on TSE patients (Aguzzi 2006). Iatrogenic CJD has additionally been caused by injection of growth hormone extracts from pituitary glands pooled from a large group of individuals that was most likely contaminated with extract from a person not diagnosed with CJD (Aguzzi et al. 2001). Other routes of infection might include blood transfusion (Llewelyn et al. 2004; Peden et al. 2004; Wroe et al. 2006) and ritual cannibalism as suggested for the disease kuru (Alpers 2008).

The physiological form of the prion protein, PrP^{C}, is highly conserved among mammals. It is a cell surface glycosylphosphatidylinositol (GPI)-anchored protein comprising an ordered C-terminal domain containing three α-helices, one anti-parallel β-sheet and a disordered N-terminal domain (Rodriguez et al. 2017; Wüthrich & Riek 2001). PrP^{C} is soluble in detergents and sensitive to protease digestion. In contrast, disease-associated PrP^{Sc} is detergent-insoluble and partly protease-resistant (Meyer et al. 1986; Prusiner 1998a). PrP^{Sc} is misfolded through posttranslational processes and has a β-sheet rich configuration, which classifies it as an amyloid fibril (Caughey et al. 1999; Pan et al. 1993; Prusiner 1998a; Riesner 2003; Rodriguez et al. 2017). Amyloids are insoluble, ordered assemblies of otherwise soluble proteins. They are composed of bundles of twisted and unbranched protein filaments that again consist of sheets of β-strands that run parallel to the filament axis forming hydrogen bonds with each other (Jahn & Radford 2005; Jucker & Walker 2013a; Vázquez-Fernández et al. 2016). Biophysicists identify amyloid fibrils by their cross-β X-ray diffraction pattern that is caused by the spacing between

β-sheets and β-strands (Eisenberg & Jucker 2012b). On the other hand, pathologists use the dye Congo Red to identify amyloid fibrils that display a typical reddish/greenish birefringence under cross-polarized light after staining (Sipe et al. 2012).

Amyloidogenic proteins in neurodegenerative diseases

The deposition of amyloid fibrils is known to be central to the pathology of more than 30 unrelated diseases including common age-related neurodegenerative diseases like Alzheimer's disease, Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD). In these diseases, just as in prion disease, the specific aggregating proteins form characteristic intracellular or extracellular lesions – and the prion paradigm is increasingly applied to explain the aggregation and progression of these otherwise physiologically soluble proteins. In amyloidosis, the aggregation process of a certain protein can either directly harm cells (called "gain of function") or the proteins that are stuck within the aggregates cannot perform their required functions anymore (called "loss of function"). The proteins involved in amyloidogenic diseases are the Aβ protein in the form of Aβ plaques and CAA in AD, the tau protein in neuronal or glial tauopathies in AD, chronic traumatic encephalopathy, frontotemporal dementia and additional neurodegenerative diseases, α-synuclein in Lewy bodies in PD, Lewy body dementia and multiple system atrophy, inclusion bodies of the huntington protein in Huntington's disease as well as superoxide dismutase 1 (SOD1) and TAR DNA-binding protein-43 (TDP-43) in ALS (Goedert 2015; Jucker & Walker 2013b; Prusiner 2013; Walker & Jucker 2015). Importantly, the mentioned proteins lack the general infectivity of prions, however they are still capable of seeded protein misfolding and the generation of self-propagating amyloid proteins. As a consequence, the "prion-like" mechanisms and prion phenomena have become a major research focus in neurodegenerative diseases and broadly influence the current understanding of neurodegeneration.

In the case of common neurodegenerative diseases, the Aβ protein was the first one to be shown to propagate in a prion-like mechanism (Kane et al. 2000; Meyer-Luehmann et al. 2006). Although the propagation of Aβ fibrils was already predicted in the 1990s by in vitro studies and inoculation studies involving nonhuman primates, it was only recently that intracranial injection studies using genetically modified rodent models showed that induction of protein deposition by a seeding mechanism within the living brain is achievable (Baker et al. 1993; Jarrett et al. 1993). These inoculation studies involve injection of brain material from either AD patients or Aβ-containing brain extract from aged transgenic mouse lines into the brain of APP transgenic mice. Thereby, the premature formation of deposits and CAA is stimulated (Kane et al. 2000; Meyer-Luehmann et al. 2006). Additionally, the process of protein deposition is inducible in transgenic animals expressing human Aβ that normally do not develop protein aggregates during their normal lifespan (Morales et al. 2012; Rosen et al. 2012). Intracranially induced Aβ deposition spreads from the injection sites to axonally connected regions and involves both neocortical as well as subcortical areas over time (Hamaguchi et al. 2012; Jucker & Walker 2013b; Ye

et al. 2015b). Aβ seeds injected into the periphery of transgenic mice can also migrate to the brain and initiate plaque deposition (Burwinkel et al. 2018; Eisele et al. 2010, 2014). This phenomenon has been linked to the onset of Aβ plaque deposition in patients receiving human growth hormone injections as children (Jaunmuktane et al. 2015; Purro et al. 2018). Initially, these cases became popular because some of the recipients developed iatrogenic CJD due to contamination of the growth hormone pool with PrP^{Sc} from donors with presymptomatic prion disase (Brown et al. 2012; Cali et al. 2018; Will 2003). Besides the development of CJD in some of these growth hormone recipients, others showed extensive accumulation of Aβ plaques and CAA in the brain most likely indicating that the growth hormone pool was additionally contaminated with Aβ seeds from presymptomatic AD patients (Duyckaerts et al. 2018; Irwin et al. 2013; Ritchie et al. 2017). Additionally, Aβ deposition in patients that were treated with dura mater transplants and later succumbed to CJD have been reported (Kovacs et al. 2016). The possibility that PrP^{Sc} cross-seeds the aggregation of the Aβ protein can likely be ruled out since PrP^{Sc} does not induce Aβ deposition in mouse models (Rasmussen et al. 2018). None of these patients developed the full clinicopathological symptoms of AD at the time of their death. However, these cases show that human transmission of Aβ pathology is possible under certain circumstances.

2.2 Aβ aggregates in human and rodent brain display heterogeneity

2.2.1 Fibrillar structures of amyloidogenic proteins

In the human AD brain, Aβ aggregates have been described as histopathologically heterogenous both within brain regions as well as among patients (Maarouf et al. 2008; Tekirian et al. 1998; Thal et al. 2006). As already described above, aggregation takes place in the vasculature in the form of CAA and in the brain parenchyma in the form of senile plaques. Regarding the parenchymal senile plaques, the two types most commonly distinguished are diffuse plaques and dense core plaques (Dickson 1997; Thal et al. 2006). Diffuse plaques start to form in the neocortical neuropil and are in general not surrounded by activated microglia and astrocytes. They can easily be distinguished from the more mature dense core plaques since they stain weakly with the amyloid-binding dyes thioflavin S and Congo Red. In comparison, dense core plaques appear to consist of either radiating or reticular compact amyloid and are positive for amyloidogenic dyes indicative of a more fibrillar plaque structure than diffuse plaques (Davies & Mann 1993; Yamaguchi et al. 1988). Furthermore, they are accompanied by microglial and astrocytic activation and synaptic loss (Yasuhara et al. 1994). A fraction of these dense core plaques is surrounded by a corona of malformed neurites. These abnormal neurites can be visualized with the same staining techniques used to identify NFTs. The plaques are called neuritic plaques and correlate with disease severity (Dickson 1997). A third form of plaque that is frequently seen in AD tissue comprises a dense core of fibrillar Aβ but without any surrounding dystrophic neurites. Those plaques have been termed "burned out plaques" and are considered to be the end stage morphologies of former neuritic plaques (Wisniewski et al. 1982). Whereas neuritic plaques are a hallmark of AD, diffuse plaques are not necessarily associated with evidence of cognitive impairment since they are commonly encountered in the brains of cognitively normal elderly individuals. Another explanation for this observation could be that diffuse plaques represent the earliest forms of plaques that develop into dense core plaques over time (Morris et al. 1996; Wolf et al. 1999). The Aβ assembly states described so far are all extracellular. However, a large amount of studies including post-mortem AD, Down Syndrome and transgenic mouse brains have recently described the intracellular accumulation of Aβ within neurons (Bayer & Wirths 2010; Cruz et al. 2018; de Kimpe et al. 2013; Friedrich et al. 2010; Zheng et al. 2012). Despite this large body of evidence indicating that Aβ accumulates intracellularly, the question to what extent this contributes to the disease remains controversial (LaFerla et al. 2007).

Detection of neurofibrillary tangles is possible with traditional histological staining methods like Bielschowsky silver stain or Thioflavin S or more recently stainings using antibodies directed against the tau protein. Neurofibrillary tangles in the AD brain occur in three distinct stages: Tangles formation starts as a "pretrangle" with tau conformers in the cell body and dendrites of neurons. Over time, aggregates appear in the perikarya and proximal cell processes and the morphology of the tangle is shaped by the type of neuron in which it forms. Eventually, the neurons die and the insoluble filaments stay in the extracellular space. These tangles are associated with microglia and astrocytes and are called

"ghost tangles". A large amount of tau burden is presented by so-called "neuropil threads" which are dendritic and axonal elements containing filamentous tau and are probably originating from NFTs (Braak et al. 1986; Mitchell & Rockwood 2000; Perry et al. 1991).

At a microscopical level, all amyloids share a common cross-β quarternary morphology that represents the thermodynamic endpoint of protein aggregation (Tycko 2015). Due to their noncrystalline, insoluble nature, it is extremely difficult to examine the specific molecular structures using the common methods for high-resolution structure determination like X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy. A detailed structural investigation of amyloids is of high importance to understand the amyloid formation process, to understand biological implications that might arise from structural variation as well as the design of compounds that might interfere with amyloid formation (Estrada & Soto 2007). Within recent years, a lot of progress has been achieved in resolving amyloid structures with rather unconventional methods including solid state NMR (ssNMR) (Tycko 2011), electron paramagnetic resonance (EPR) (Margittai & Langen 2008), electron microscopy (Goldsbury et al. 2005) and cryo-electron microscopy (Meinhardt et al. 2009).

A typical amyloid fibril comprises 5-15 nm in width, is unbranched and straight and can approach many microns. X-ray fiber diffraction of aligned amyloids represents a typical diffraction pattern with a meridional reflection at 4,7 A and an equatorial reflection at 8-11 A which is considered to represent the structural organization of the cross-β-sheet motif (Eanes & Glenner 1968; Sunde & Blake 1997). Based on the X-ray pattern, individual β-sheets are comprised of multiple repeating β-strands that are aligned perpendicular to the fibril axis (4,7 A inter-strand spacing) and connected by hydrogen bonds (Sunde & Blake 1997). Two or more β-sheets can align with each other (8-11 A inter-sheet spacing) and run parallel to the fibril axis connected by sidechain-sidechain interactions (Nelson et al. 2005; Sawaya et al. 2007). This highly ordered packing of the proteins results in a repetitive arrangement of thousands of protein copies that can be visualized with EM (Astbury et al. 1935). The hydrogen bonds between the β-strands as well as hydrogen bonds between amides (glutamine and asparagine) within the side chains lead to an extremely high stability of the β-fibrils (Tsemekhman et al. 2007). Sidechains between two or more β-sheets are additionally tightly interdigitated and are called steric zippers. Since the gap between two β-sheets is devoid of water molecules, those motifs are called dry steric zippers. This hydrophobic effect contributes to the overall fibril stability (Eisenberg & Jucker 2012a; Riek & Eisenberg 2016).

One of the most fascinating characteristics of amyloid formation is the structural polymorphism of a single polypeptide that can vary due to different growth conditions and is not determined by its amino acid sequence (Petkova et al. 2006). β-strands within an amyloid fibril can either have a parallel, an anti-parallel or a hairpin orientation which means that β-strands can run in the same direction or in opposing directions. Furthermore, those β-strands usually run "in-register" which means that strands

align with each other so that identical sidechains intercalate along the fibril axis (Eisenberg & Jucker 2012a). The β-sheet to β-sheet arrangement in steric zippers is most common face-to-face but other arrangements like face-to-back, opposite edges up or antiparallel strand arrangement(Sawaya et al. 2007). Some amyloid fibrils can even contain more than just one steric zipper since many proteins contain more than one potential steric zipper forming segment (Colletier et al. 2011; Lewandowski et al. 2011). Summed up, 3 different types of polymorphism can be distinguished: The "packing polymorphism" which is based on a different orientation between the β-strands, the "segmental polymorphism" which implies that distinct segments of an amyloid protein can be involved in the β-sheet structure as well as the "assembly polymorphism" which states that the packing of individual β-sheets forming a fibril is different although the β-strand packing might be identical (Riek & Eisenberg 2016) (**Figure 3**).

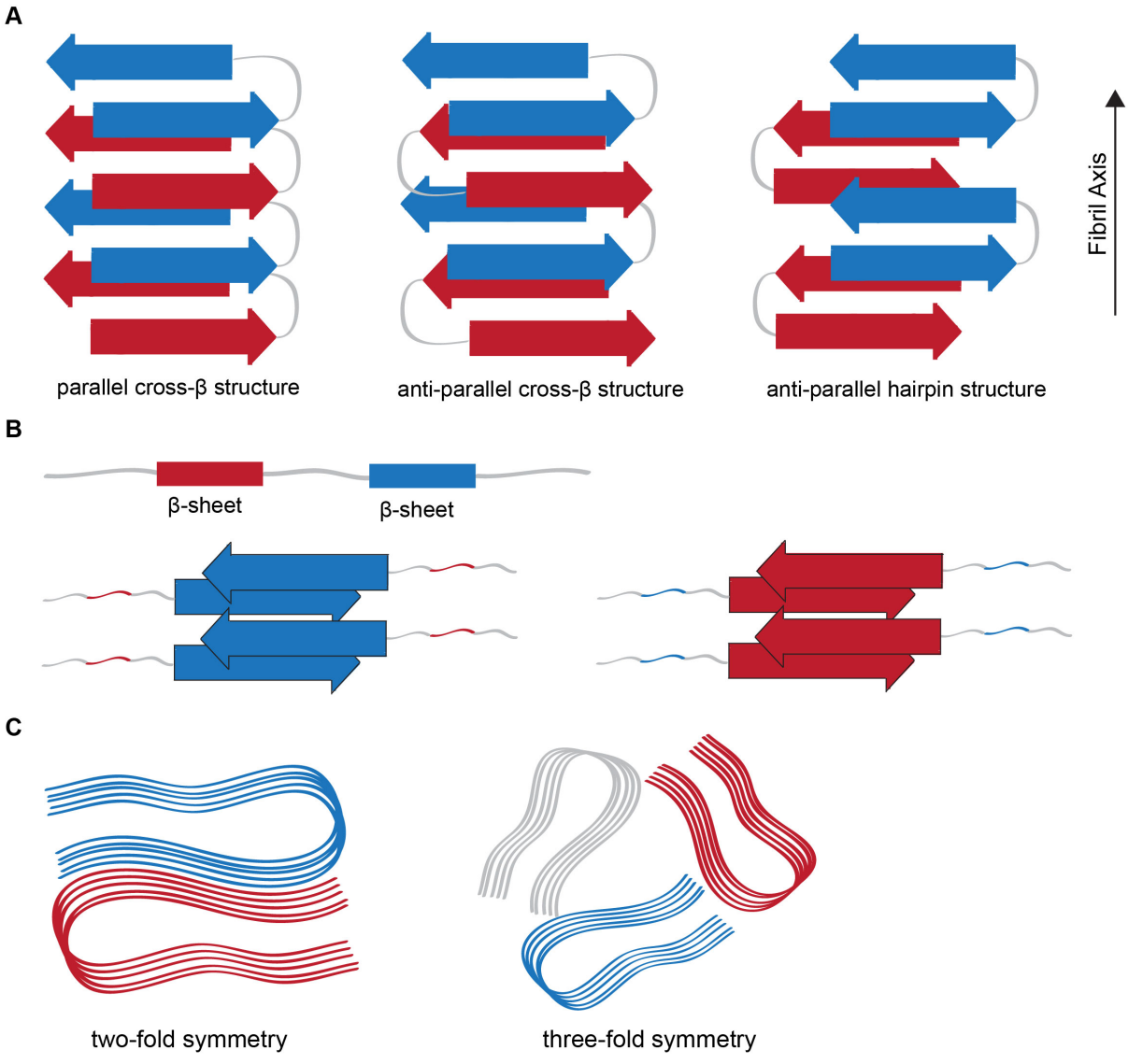

Figure 3. Structural polymorphisms of amyloidogenic proteins. A) Packing polymorphisms include parallel cross-β structures, in which the β-strands of adjacent peptides are organized in parallel of the fibril axis; anti-parallel cross-β structures, in which the β-strands of adjacent peptides run in opposite directions; as well as anti-parallel β-hairpin structures. **B)** Segmental polymorphism involves the variability of core-forming β-strands within the same peptide. **C)** Structural polymorphs describe the overall symmetry of whole amyloid fibrils to each other. In the case of the Aβ protein, both two-fold as well as three-fold symmetries have been described.

The protein environment substantially influences the fibril morphologies a given protein can obtain. External factors such as pH value, temperature, agitation or salts determine the protein structure as shown in a variety of publications (Klement et al. 2007; Makarava & Baskakov 2008; Pedersen & Otzen 2008; Petkova et al. 2005; Toyama et al. 2007; Verel et al. 2008). Some fibril structures can propagate by seeding and extension of structural templates. Thereby, the fibril morphology is obtained and propagated to physiological forms of the protein (Peim et al. 2006). Interestingly, even under the same environmental conditions and within the same sample, considerable variations within the fibril morphologies may exist which indicates that a specific physico-chemical environment can favor a specific fibril structure but does not necessarily lead to one dominant structure(Fändrich et al. 2009).

Regarding the Aβ peptide, full molecular structures are mostly based upon ssNMR measurements in combination with TEM (Bertini et al. 2011; Paravastu et al. 2008a; Petkova et al. 2006; Schütz et al. 2015; Sgourakis et al. 2015; Xiao et al. 2015). Structures mostly rely on *in vitro* Aβ fibrils since the preparation and structure determination of Aβ deposits from AD patient tissue is currently not straightforward –Aβ fibrils are often neither abundant enough nor homogeneous and pure enough. For instance, *in vitro* studies of the $A\beta_{40}$ peptide could show that the protein gives rise to a variety of differently structured amyloid fibrils as seen by their appearance in TEM (Chamberlain et al. 2000; Goldsbury et al. 2011; Meinhardt et al. 2009). Initial ssNMR measurements on *in vitro* derived $A\beta_{40}$ peptides indicated that the Aβ peptide is both capable of a parallel as well as an anti-parallel β-sheet arrangement (Benzinger et al. 1998; Colletier et al. 2011; Lansbury et al. 1995). Petkova et al. then showed that the $A\beta_{40}$ peptide can form into a variety of different morphologies by variations of in vitro growth conditions. Fibrils grown at 24°C and pH 7,4 including gentle agitation gave rise to a so-called "striated-ribbon" morphology. Fibrils grown at 24°C and pH 7,4 but without agitation showed a "twisted" morphology. The difference in morphologies as seen in TEM were confirmed by ssNMR and led to full molecular structural models for both fibrils. According to ssNMR results, the peptide conformations within both polymorphs are in general quite similar: Residues 1-9 of the $A\beta_{40}$ peptide display a disordered segment, followed by a β-strand segment between residues 10 and 22, separated by a bend or loop at residues 23-29 and a second β-strand segment between residues 30 and 40. The β-strands are aligned in parallel and in-register and interact by hydrophobic amino acid sidechains. The overall difference lies within the number of β-sheets interacting with each other. While the "striated-ribbon" morphology consists of a two-fold rotational symmetry around the fibril growth axis, the

"twisted" morphology contains a three-fold rotational symmetry (Paravastu et al. 2008b; Petkova et al. 2005, 2006). According to several studies, the number of currently known distinct $A\beta_{40}$ polymorphisms is at least five (Bertini et al. 2011; Kodali et al. 2010; Lu et al. 2013; Meinhardt et al. 2009; Niu et al. 2014). Full structural models of $A\beta_{42}$ based on ssNMR do currently not exist. However, fibrils that were prepared in vitro show similar morphologies to $A\beta_{40}$ fibrils: they contain parallel in-register β-strands interacting through hydrophobic contacts and exist as two-fold or three-fold symmetric polymorphs (Antzutkin et al. 2002; Lührs et al. 2005; Olofsson et al. 2007; Sato et al. 2006; Török et al. 2002).

Whether the Aβ fibril polymorphisms created *in vitro* represent the fibril structures that deposit in the AD brain is debatable. To take a closer look at *in vivo* fibril structures, Tycko and colleagues developed a technique to examine AD postmortem brain extracts via NMR spectroscopy. To generate enough fibril material, the extracted fibril material was used as a seed to propagate its structure on monomeric $A\beta_{40}$ protein. Using this technique, the researchers could investigate the amyloid structures of two clinically heterogeneous AD patients and showed that both patients harbored different $A\beta_{40}$ fibril structures. While the tissue-derived Aβ fibril showed a threefold-symmetry, it revealed considerable structural differences in comparison to fibrils obtained from recombinant protein (Lu et al. 2013). Another recent study used a gentle purification procedure to isolate Aβ fibrils from the meninges of three AD patients and subsequent structure analysis using cryo-EM. The observed fibril structures consist of dimers of C-shaped β-sheets positioned back-to-back and twisting to the right. In total, each peptide comprised 4 β-strands that form the C-shape of the protein. The right-twisted conformation is in harsh contrast to the left-handed Aβ fibril conformations as seen in structures derived from recombinant peptides (Kollmer et al. 2019). The studies mentioned above emphasize the importance of fibril structure determination of diseased AD tissue, whereas structures observed in experiments using synthetic or recombinant Aβ peptides should be considered carefully.

Aβ fibrils are often visualized with dyes such as Congo Red and Thioflavin S that bind very specifically to amyloids however they do not give any further information about conformational characteristics (Klingstedt et al. 2011). With the recent development of new amyloid binding dyes, called luminescent conjugated oligothiophenes (LCOs), it is now possible to distinguish between different fibril structures (Klingstedt et al. 2013). LCOs have a conjugated thiophene backbone and emit strong fluorescence upon binding to aggregates. When compared to conventional amyloid-bindings dyes, LCOs have a higher sensitivity and identify a wider range of amyloidogenic aggregates (Klingstedt & Nilsson 2012). ssNMR has revealed the operating principles of LCOs in distinguishing between different aggregate structures. LCOs bind to grooves along the filament axis of aggregates and interact with charged amino acids within these grooves (Herrmann et al. 2015). Depending on the aggregate structures the LCOs bind to, the colour of emitted light changes. As a consequence, distinct protein assemblies can be separated based on the colour of the LCO. This new application has already been successfully used to discriminate

between different prion strains (Sigurdson et al. 2007) and could recently differentiate α-synuclein assemblies between PD and MSA (Klingstedt et al. 2019).

2.2.2 Amyloid-β plaques display distinct clouds of conformations

In reference to:
Rasmussen J*, Mahler J*, **Beschorner N***, Kaeser SA, Häsler LM, Baumann F, Nyström S, Portelius E, Blennow K, Lashley T, Fox NC, Sepulveda-Falla D, Glatzel M, Oblak AL, Ghetti B, Nilsson KPR, Hammarström P, Staufenbiel M, Walker LC, Jucker M. Amyloid polymorphisms constitute distinct clouds of conformational variants in different etiological subtypes of Alzheimer's disease. Proc Natl Acad Sci USA 2017; 114; 13018-13023.
(dio:10.1073/pnas.1713215114)
*equal contribution

The term strain comes from prion biology and is defined as conformationally distinct infectious prion isolates that exhibit specific prion-disease characteristics when transmitted to identical hosts over several passages. These characteristics encompass specific incubation times, histopathological lesions and neuronally involved areas. In general, phenotypic characteristics persist upon serial transmission. The strain-specific properties are likely due to different disease-associated PrP^{sc} conformations that give rise to the different disease characteristics (Aguzzi et al. 2007; Safar et al. 1998). Evidence that PrP^{Sc} exists in multiple conformations comes from biochemical studies showing that proteinase K resistant cores of different PrP^{Sc} types display distinct electrophoretic mobility. This is likely to result from the exposure of different protein sites for enzymatic cleavage (Bessen & Marsh 1994; Bessen et al. 1995). The N-terminus of human prion protein contains multiple proteinase-K digestion sites. Subtypes of sporadic CJD show different proteinase-K resistant core sizes that could be caused due to slightly altered structures of the N-terminus (Parchi et al. 2000; Zanusso et al. 2004). Quite often, the distinct biochemical signatures of PrP^{Sc} match up with a specific disease phenotype. Of importance, multiple PrP^{Sc} isoforms seem to co-occur in CJD types (Bartz et al. 2007; Nishida et al. 2005). Data suggest that upon injection of two prion strains into susceptible hosts, one impedes the ability of the other (Dickinson et al. 1972; Manuelidis 1998). Another important characteristic of prion strains is the so called species barrier according to which prions isolated from one animal species are less infectious in another animal species as apparent by longer incubation times and reduced neurological toxicity. Such a species barrier occurs between mice and hamsters and is probably due to different host PrP sequences that slow down or even stop the templated conversion process (Race et al. 2002). Upon re-injection into the original host, the full infection potential can rapidly return which indicates that the PrP agent can non-pathologically replicate for a long time within an unsuitable host without losing its toxicity (Hill et al. 2000, 2003). In some cases, the species barrier is reduced over time resulting in decreased incubation times – this phenomenon is called adaptation (Race et al. 2002; Sigurdson et al. 2006). The mechanism

behind the adaptation phenomena is of high interest to prion research and currently there are two hypotheses being discussed: The "cloud hypothesis" due to which PrP^{Sc} particles consist of an intrinsically heterogeneous pool of strains. After a cross-species transmission, a minor fraction of PrP^{Sc} strain may become dominant in the new host over time and cause a new disease phenotype (Collinge & Clarke 2007; Li et al. 2010). The other hypothesis is called the "deformed templating model" and postulates that a change in replication environment actively generates new PrP^{Sc} strains. A new strain that fits well to the new environment will eventually accumulate after multiple trial-and-error seedings events (Makarava et al. 2011, 2012, 2015).

The identification that prion protein architecture and not amino acid sequence dictates the pathobiology of these molecules is of high importance for other neurodegenerative diseases that are caused by protein aggregation. Similar to the variety of prion diseases, AD has been described as a heterogeneous disease. Variability in cognitive symptoms, age of onset and the general rate of decline describe the heterogeneity of AD (McKhann et al. 2011). Neurologists roughly divide AD into several subtypes: typical AD as a late-onset syndrome with an amnestic impairment involving memory functions and other cognitive domains due to hippocampal and temporal-parietal atrophy (Dubois et al. 2007). The temporal variant of AD is characterized as a late-onset syndrome with isolated episodic memory impairment and a slow decline over time (Butters et al. 1996). Atrophy is limited to the mesotemporal lobes and visuospatial and executive functions remain almost normal (Marra et al. 2012). Several subtypes comprise a language variant of AD, often of an early onset with non-fluent speech caused by atrophy to the left perisylvian region (Alladi et al. 2007; Galton et al. 2000; Gorno-Tempini et al. 2008; Green et al. 1990). Visuoperceptive variants of AD, among them posterior cortical atrophy (PCA), display visuospatial dysfunctions with only subtle memory impairment associated with a right hemisphere pathology and atrophy (Chase et al. 1984; Fisher et al. 1997; Tang-Wai et al. 2004). The extremely rare frontal variant of AD is associated with frontal cognitive and behavioral symptoms (Alladi et al. 2007; Johnson et al. 1999). As already described in detail, the Aβ protein can aggregate into strain-like structures. Therefore, it seems tempting to correlate distinct neurodegenerative phenotypes with different strain-like fingerprints. To determine the potential of structurally different Aβ deposits to persist upon serial transmission, seeding experiments with two transgenic mouse lines, APP23 and APPPS1 harboring phenotypically distinct plaque morphologies and distribution patterns, were conducted. Intracerebral inoculation of brain homogenate of one mouse line into the other resulted in plaque morphologies resembling the donor material although some influence of the endogenous host material was visible as well. When injected brain material was stained with LCOs, the fluorescence spectra of seeded plaques differed from the endogenous plaques of the mouse model (Heilbronner et al. 2013; Meyer-Luehmann et al. 2006). Some of the strain-like properties of the Aβ protein have been proposed to be traced back to differences in the $A\beta_{40}/A\beta_{42}$ ratios. In another study, aggregates grown from either synthetic $A\beta_{40}$ or $A\beta_{42}$ produced distinct morphological fibril characteristics *in vivo* (Stöhr et al. 2014)

In order to extent the effects of plaque polymorphism to human AD patients, important results have been obtained over the past years. A study that focused on forms of AD with an accelerated disease course, called rapidly progressive Alzheimer's disease (rpAD), concluded that these forms of the disease comprise a significantly higher level of less stable Aβ fibrils when compared to typical forms of the disease as shown by a variety of biochemical stability assays. The authors of this study therefore concluded that patients with rpAD have a unique structural fibril organization (Cohen et al. 2015). Fibril structures of AD patients were further evaluated in two pioneering studies from the Tycko lab. The first study, carried out by Lu et al. in 2013, compared fibril structures from two patients that differed in clinical history, namely AD with progressive aphasia and mild cortical atrophy versus typical AD with severe cortical atrophy. Extracted brain tissue was used to seed synthetic fibril growth of $A\beta_{40}$ *in vitro*. Both TEM images and ssNMR revealed two distinct predominant $A\beta_{40}$ fibril structures (Lu et al. 2013). A follow-up study investigating a larger cohort of patients including rpAD, PCA-AD and typical forms of AD using the same experimental setup revealed that the same predominant $A\beta_{40}$ fibril structure exists both in PCA-AD and typical AD but differs from rpAD. In contrast, brain extracts used to seed synthetic $A\beta_{42}$ showed a high structural heterogeneity among all patient samples (Qiang et al. 2017). Importantly, these structures were not shown to propagate in transgenic mouse models, a characteristic that is crucial for the detection of strains (Aguzzi et al. 2007).

To further advance the characterization of different Aβ fibril structures in human AD patients, our recent study made use of LCOs to investigate plaque structures in a large cohort of AD patients with various etiologies (Rasmussen et al. 2017). LCOs can be directly applied to fresh frozen human brain tissue. This enables the investigation of structural fibril features within their native tissue environment and thereby bypasses the selection of certain dominant structures which might occur during the preparation for ssNMR analysis. The combination of the two LCO dyes h-FTAA and q-FTAA was applied to discern the plaque morphologies of both fAD cases (APP-V717I, PSEN1-A431E, PSEN1-E280A and PSEN1-F105L) and sAD cases including PCA patients (Nyström et al. 2013). The analysis was solely performed on the dense cores of the Aβ deposits to restrict the analysis to the β-sheet structures of the protein. In all cases, temporal, occipital and frontal cortex samples were investigated to explore regional brain differences. There were no spectral plaque differences within the three individual brain regions of single patients, as in agreement with various other studies (Cohen et al. 2015; Lu et al. 2013; Qiang et al. 2017). On the other hand, obvious spectral differences between the groups of fAD patients and between the sAD patients and PCA cases were detected. Of importance, even within the group of sporadic patients, considerable plaque variation among patients was visible. One unique sporadic case that was described to display a low binding of PiB also showed a specific LCO emission spectrum when compared to other sporadic cases (Rosen et al. 2010). At a single patient level, it was observed that the spectral signatures of individual plaques can greatly vary, including the emission spectra of plaques that were in close

proximity to each other. Interestingly, the highest variability at a single patient level was discovered within some of the sporadic patients. These results were referred to as clouds of conformations, in analogy to clouds of prion conformations. This term has been used to explain the heterogeneous nature of prion samples and is used to explain the strain adaptation that is observed during transmission between different animal species (Collinge 2010). In the case of AD, these results hypothesize that instead of only one plaque conformation per patient, several subtypes of conformations exist that may cluster around a dominant fibril variant. Furthermore, the spectral properties of sAD and PCA were compared to clinical phenotypes (Age, ApoE status and postmortem interval) as well as biochemical characteristics ($A\beta_{40}/A\beta_{42}$ ratio, Aβ concentration as well as proteinase-K resistance) but no correlation could be observed. In a next step, the strain-like transmission properties of APP-V717I, PSEN1-A431E, sAD and the PiB-negative case were assessed by injection into APP23 mice. Therefore, the brain material was diluted to the same concentration prior to injection. Results indicate spectral emission characteristics similar to the injected material and the amount of induced aggregation differed between the injected groups. However, spectral properties appear less distinct than the initial brain material which might be due to the interference with endogenous plaque deposition in APP23 mice as described before (Heilbronner et al. 2013; Meyer-Luehmann et al. 2006; Watts et al. 2014).

Although the focus of this study was on the Aβ protein, we additionally examined the spectral characteristics of the tau protein. Spectral analysis was restricted to neurofibrillary tangles. Of importance, there was no detectable difference of tau fibril characteristics - neither among patient subtypes nor within individual patients. These data agree with recent cryo-EM data implicating that tau fibrils in AD patients do not vary however that different diseases involving tau aggregation, like AD and Pick's disease, display different fibril polymorphisms (Falcon et al. 2018; Fitzpatrick et al. 2017).

This study is the first to investigate the structural properties of Aβ plaques within the native environment of the brain parenchyma. The results indicate that subtypes of AD patients contain similar predominant Aβ fibril structures that can be transmitted into susceptible hosts. Therefore, it is tempting to speculate that these fibril polymorphisms present distinct Aβ strains and are accountable for the differences in clinical phenotypes seen in AD patients, especially in the cases of sAD and PCA (Crutch et al. 2017). These results are in accordance with two additional studies, where distinct conformations of the $A\beta_{42}$ peptide were observed and correlated with distinct rates of cognitive decline (Cohen et al. 2015; Qiang et al. 2017). Another implication of these data relates to diagnostics and treatment of the disease. Variations in amyloid structures are very likely to complicate the outcome of current drug trials using antibodies to clear Aβ fibrils from the brain of AD patients. Especially monoclonal antibodies would fail to recognize the full range of aggregate polymorphisms existing within a brain and among AD patients. In this case, a polyclonal mixture of different antibodies would be more successful in treatment.

It is important to note that studies analyzing Aβ fibril conformations solely examine end stages of the disease. It is likely possible that fibril structures change over the course of the disease and that end stage protein aggregates do not display the same characteristics as earlier ones. In terms of medical interventions and insights into the causes of the disease, it is important to investigate the earliest aggregates that give rise to the illness. However, the detection of the very first aggregate seeds in AD is currently technically very difficult. Therefore, it is reasonable to investigate if end stage Aβ fibrils can provide information about the nature of the first aggregates.

2.3 Exploring the seeding paradigm

2.3.1 Mechanisms of protein seeding

A protein that misfolds and subsequently escapes from normal clearance pathways can start a pathogenic process in which the protein aggregates progressively into intracellular or extracellular deposits. New insights into the process of protein aggregation come from the self-propagating characteristics of the prion protein. According to the "prion paradigm", misfolded PrP proteins aggregate with each other and impose their structures on soluble PrP peptides. As a consequence, prions act as templates, or seeds, that give rise to a self-propagating chain-reaction that spreads within the nervous system (Aguzzi 2009; Caughey et al. 2009; Collinge 2001; Prusiner 1998b).

The process of amyloid formation displays a typical sigmoidal reaction time course. This time course involves an initial lag phase that is observed before a rapid growth phase as a general feature of nucleated polymerization (Serio et al. 2000; ten Wolde & Frenkel 1997). If the quantity of the aggregating protein is limited, a plateau phase follows on the growth phase as a result of the depletion of soluble protein species. *In vitro* studies suggest that the formation of amyloidogenic proteins starts with a slow nucleation phase that is equivalent to the aggregation of the protein into a seed. The aggregation process probably goes through a series of intermediate states until the initial seed is formed (Jerrett & Lansbury 1993; Lee et al. 2011). The initial formation of an aggregate from a soluble protein is defined as "primary nucleation", a process that is considered spontaneous. In the case of amyloidogenic proteins, the lag phase can also involve "secondary nucleation" events. Secondary nucleation involves fibril fragmentation that increases the number of fibril ends that can attach soluble proteins as well as surface catalyzed secondary nucleation through the formation of new nuclei by already existing aggregates. In this case, the time of the lag phase is not primarily dependent on the first seeds being generated through primary nucleation but on the amplification by secondary nucleation steps until a level is reached that can be detected in protein assays (Cohen et al. 2012; Knowles et al. 2009). Microscopic and mass spectrometric techniques as well as single-molecule optical methods could show that the initial stages of protein aggregation are characterized by the formation of various oligomeric peptide structures. In the case of PrP, the oligomers perform a slow transition from initially relatively disorganized structures to more and more compact ones with a rudimentary β-sheet structure before they grow into fibrillar

species (Bernstein et al. 2009; Narayan et al. 2012; Nettleton et al. 2000; Serio et al. 2000; Smith et al. 2010)

During the growth phase of amyloid formation, the overall conversion rate of the protein into its amyloid form is greatest. The onset and end of the growth phase can be quite sharp depending on the underlying mechanism (Cohen et al. 2012). For amyloidogenic proteins, it is considered that during the phase mainly secondary nucleation processes play a role. Here, the ends of the initial seeds are extended by monomers or oligomers of the peptide by conformational conversion. A growing fibril can eventually break and thereby the amyloid formation becomes self-propagating due to the spreading of new seeds to distinct brain areas (Knowles et al. 2014) (**Figure 4**).

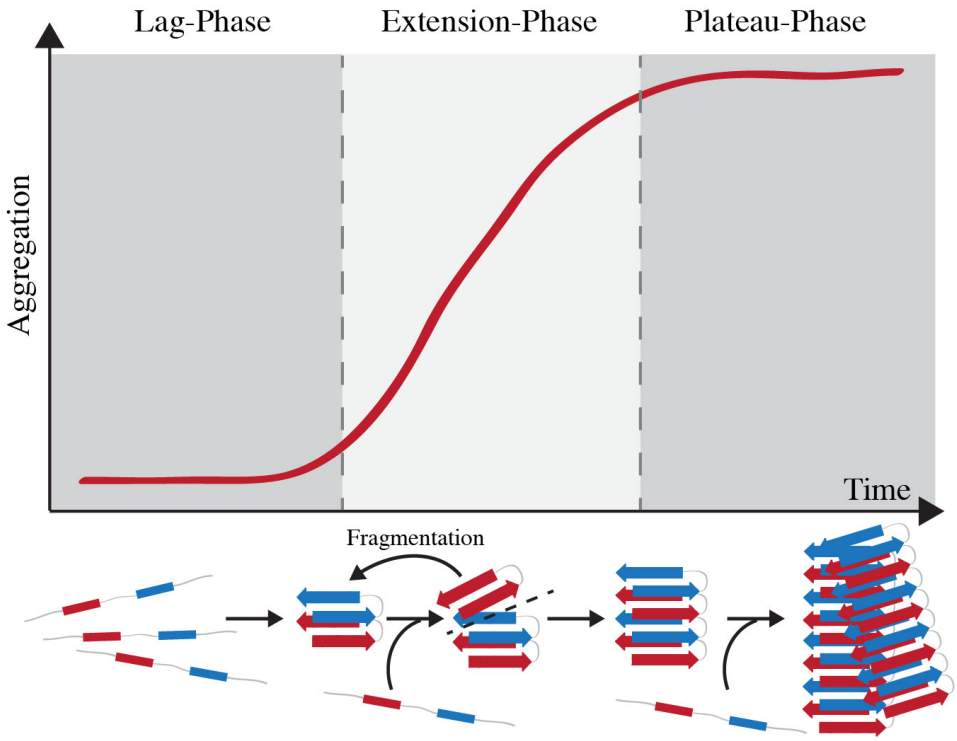

Figure 4. The process of amyloid formation divided into three distinct phases. The process of amyloid aggregation displays a typical sigmoidal reaction time course. This time course involves an initial lag phase that is observed before a rapid growth phase as a general feature of nucleated polymerization If the quantity of the aggregating protein is limited, a plateau phase follows on the growth phase as a result of the depletion of soluble protein species. The lag phase involves a slow nucleation phase that reflects the aggregation of the protein into a seed, equivalent to primary nucleation. The aggregation process probably goes through a series of intermediate states until the initial seed is formed. During the extension phase, secondary nucleation processes involving fibril fragmentations that increase the number of fibril ends become more important and cause the rapid fibril growth.

Data supporting the prion paradigm mainly come from *in vitro* studies analyzing the aggregation kinetics. Regarding the prion protein, PrP^{Sc} is very low to undetectable compared to endogenous PrP^{C} at the time of infection. As soon as the animal shows first clinical signs of the disease, the amount of PrP^{Sc} significantly increased. These observations suggest that PrP^{Sc} replicates *in vivo* utilizing the soluble protein. This hypothesis is supported by the fact that PrP knockout animals are resistant to infection with PrP^{Sc} particles (Büeler et al. 1993). The method Protein Misfolding Cyclic Amplification (PMCA) has been very valuable to mimic the PrP^{Sc} conversion process taking place *in vivo*. The method makes use of the addition of stable seeds to the sample for acceleration of the nucleation process and for shortening the lag phase in a process termed seeding (Harper et al. 1997; Jarrett & Lansbury 1992; Jarrett et al. 1993). It consists of several cycles of accelerated protein aggregation and each cycle is composed of two phases: During phase 1, the sample contains very low amounts of PrP^{Sc} and high amounts of PrP^{C} being incubated together to induce the formation of PrP^{Sc}. During the second phase, the samples is sonicated for breakage of fibrils thereby generating new seeds. After each cycle, the number of PrP^{Sc} increases exponentially and can be visualized by Western Blot or ELISA (Saborio et al. 2001; Soto et al. 2002).

Another commonly used kinetic assay for amyloidogenic proteins involves the usage of ThT fluorescence which show a linear increase in fluorescence related to the total mass of generated fibrils (Cohen et al. 2013). Meisl et al. could show for the $A\beta_{42}$ peptide that once a low but critical concentration of around 10nM by primary nucleation is reached, further fibrillar species are formed through secondary nucleation steps. This promotes a positive feedback loop that may explain the aggregation-prone characteristics of the $A\beta_{42}$ peptide. In contrast, the $A\beta_{40}$ peptide displays a prolonged lag phase and the contribution from primary nucleation is decreased relative to $A\beta_{42}$ and the aggregation of the peptide rather depends on secondary nucleation processes. One explanation for the difference in the peptide behavior might be the increased hydrophobicity imposed by residues Ile41 and Ala42 that leads to more spontaneous primary nucleation events of $A\beta_{42}$ (Meisl et al. 2014).

In vivo evidence for the prion paradigm comes from inoculation studies using various amyloidogenic proteins to seed the aggregation of endogenous soluble peptides (Korenaga et al. 2006; Westermark & Westermark 2010; Yan et al. 2007; Zhang et al. 2008). *In vivo* aggregation of the Aβ protein can be induced by the intracerebral injection of minute amounts of Aβ-containing brain extracts from AD patients or aged APP-transgenic rodents that triggers the accelerated formation of plaques and CAA in these models (Jucker 2010; Kane et al. 2000; Meyer-Luehmann et al. 2006). Importantly, the induction of Aβ deposition is further possible in transgenic mice that do not develop Aβ plaques within their normal lifespan thereby excluding that the aggregation process seen in depositing mice is only enhanced by infusion of Aβ fibrils (Morales et al. 2012; Rosen et al. 2012). While Aβ from brain homogenate is extremely effective in seeding lesions, injection of synthetic human Aβ fibrils is less potent in inducing

aggregation, similar to the results that have been obtained for recombinant prion protein (Legname et al. 2004; Stöhr et al. 2014). The denaturation of proteins, the selective removal of Aβ and the active or passive immunization of mice against Aβ complete negates or diminishes the ability of the injectate to seed Aβ aggregation (Meyer-Luehmann et al. 2006).

Owing to their high stability due to the amyloid state, Aβ seeds as well as PrP prions are extremely resistant to cellular and physicochemical degradation. These characteristics contribute to their persistence and, in the case of the prion protein, to their infectivity (Taylor 1999). The inactivation of Aβ seeds has been extensively studied concluding that some amyloidogenic forms of the protein are resistance to heat-inactivation, formaldehyde-fixation and protease degradation (Fritschi et al. 2014; Langer et al. 2011). Furthermore, there is evidence that Aβ seeds survive for at least 6 months in rodent brain without replication and are able to regain seeding-activity upon re-injection in susceptible transgenic mice (Ye et al. 2015a). In comparison, prion seeds have been reported to stay infectious after incubation times of almost 20 months in hosts not expressing PrP (Diack et al. 2017). However, it should be mentioned that an analysis of the general Aβ-inducing agents revealed that small, oligomeric seeds harbor a high amyloid-inducing activity when injected into transgenic mice. These oligomeric seeds are prone to inactivation by proteinase-K digestion, a characteristic that is comparable to the proteinase-K sensitive oligomeric forms of PrP (Fritschi et al. 2014; Langer et al. 2011; Tzaban et al. 2002).

2.3.2 Nature and durability of Aβ seeds

In reference to:
Beschorner N, Ye L, Häsler LM, Jucker M. Persistence of amyloid-β plaque polymorphism in APP null mouse brain is mediated by the $A\beta_{42}$ isoform. *in preparation*

The question which Aβ species in the brain is essential for the seeding activity observed and what kind of co-factors are required still remains elusive. Langer et al. could show that Aβ seeds are not homogenous forms of Aβ aggregates but rather a mixture of both small soluble and insoluble as well as proteinase-K sensitive and proteinase-K resistant Aβ species. These results were obtained by the injection of 100,000 x g ultracentrifuged supernatant or pellet fraction treated with or without proteinase-K into susceptible transgenic mice and the amount of Aβ deposition was used as a readout of seeding activity. Another interesting finding from this study revealed that sonication which induces fragmentation of insoluble fibrils into smaller and soluble Aβ seeds in general enhanced the seeding activity of the injected homogenate (Langer et al. 2011). This finding is consistent with results from earlier fragmentation studies demonstrating that Aβ oligomers are of particular importance for the initiation of aggregation during the earlier phases of the seeding process (Falsig et al. 2008; Jarrett et al. 1993; Katzmarski et al. 2020; Knowles et al. 2009; Xue et al. 2010). Along with these findings, another

study implicated that the Aβ seeding potential of brain extracts is highest during the very early stages of cerebral amyloidosis (Ye et al. 2017).

Apart from the different Aβ variants that emerge during the seeding process, the two most abundant Aβ species, Aβ40 and Aβ42, display different characteristics. Aβ42 is known to be more hydrophobic resulting in a higher potential to aggregate into oligomeric species than the more abundant Aβ40. This tendency is mainly evoked by the hydrophobicity of the C-terminus. Although these differences are quite small, Aβ42 reflects a dramatically increased propensity to form amyloid fibrils (Bitan et al. 2003; Luhrs et al. 2005). Strain-like differences in two mouse models, APP23 and APPPS1, were partly ascribed to the differences in Aβ40/Aβ42 ratios. These mouse lines develop morphologically different plaques that show distinct LCO emission spectra as well as either high or low Aβ40/Aβ42 ratios. Upon seeded transmission into the respective other mouse line, polymorphisms could be propagated and differed from endogenous unseeded plaques. Aβ40/Aβ42 ratios also resembled the injected material (Heilbronner et al. 2013). These findings were extended in an *ex vivo* hippocampal slice culture model. In this setup, the morphology and LCO emission spectra of seeded plaques were influenced by the Aβ species added to the medium (Novotny et al. 2016). Another study made use of synthetic Aβ40 and Aβ42 fibrils generated in vitro that gave rise to polymorphic strain-like deposits that could be propagated upon in vivo passage (Stöhr et al. 2014). In contrast, amyloid deposition in mice has been shown to be almost completely driven by Aβ42 deposition (McGowan et al. 2005). The fact, that the Aβ42 is also the principal protein species that deposits in parenchymal senile plaques within the brain and the relative ratio of Aβ40/Aβ42 is closely related to the age of disease onset in familial AD and a higher neurotoxicity, questions the relevance of the Aβ40 peptide within human AD patients (Duering et al. 2005; Selkoe 2001).

To investigate the nature of the Aβ seed and the influence of the Aβ40 and Aβ42 species on seeding and strain-like properties, our study made use of the already described APP23 and APPPS1 mouse models that differ significantly in their morphology, LCO spectral plaque emission and Aβ40/Aβ42 ratios (Heilbronner et al. 2013). As already reported above, studies could show that the Aβ seed can be extremely long lasting in the living brain: a pool of brain homogenate from aged APP23 transgenic mice was injected into the hippocampi of APP-knockout mice. These mice do not express APP and are therefore incapable of replicating the injected Aβ seeds and the injectate was therefore subject to cellular and extracellular degradation. Even after a total of six months of incubation, some of the seeding-competent Aβ remained in the recipients although undetectable by currently available methods and regained seeding potential upon secondary transmission into APP transgenic animals. The Aβ concentrations in APP-knockout animals could be detected within the first seven days and were below detection in the extract-injected hippocampi within 30 days using electrochemiluminescence-linked (ECL) immunoassay (Ye et al. 2015a).

For studying the *in vivo* degradation of Aβ polymorphisms, a pool of brain material either containing aged APP23 material or aged APPPS1 material was injected into APP-knockout mice. Residual Aβ concentrations were measured at different time points for up to 180 days post injection using an ultra-sensitive bead-based single-molecule array Simoa technology. Astonishingly, for both brain homogenates, the Aβ40 species was cleared from the brain within 30 days post injection, whereas the Aβ42 species could be detected for up to 60 days post injection and partially for up to 180 days post injection. These results suggest that the Aβ42 species is by far more resistant to cellular degradation mechanisms as compared to the Aβ40 species. Following the measurement of the residual human Aβ concentration in the injected hippocampi, we investigated if the APP-knockout brain homogenate can still induce the morphological and biochemical characteristics as seen in the APP23 and APPPS1 mouse strain by injection into young APP23 transgenic mice. APP23 and APPPS1 homogenate that was incubated in APP-knockout animals for up to 180 days could still induce the typical morphological strain-like plaque characteristics of the specific mouse lines. Additionally, the emitted spectral emission by LCO binding was comparable to the injected host material and revealed a significant effect between the injected groups. However, the APP23 transgenic mice that were injected by secondary transmission revealed the typical high Aβ40/Aβ42 ratio of the APP23 mouse strain and therefore did not reflect the ratio of the individual injected material. These results challenge previous studies implicating that the Aβ40/Aβ42 ratio is maintained by intracranial transmission studies and is the dominant factor that gives rise to strain-like morphologies both in *in vitro* studies using recombinant protein as well as in *in vivo* seeding in rodent models (Heilbronner et al. 2013; Novotny et al. 2016). Instead of a selection mechanism whereby the seeded Aβ deposits mainly incorporate the same Aβ species resembling the seed, these results suggest that the Aβ42 seed is able to cross-seed the more abundant Aβ40 species and nevertheless retains its strain-like morphology. One explanation for the findings in earlier studies might be that in primary transmission studies that comprise the original Aβ40/Aβ42 ratio of the injected material, Aβ40 preferentially seeds the Aβ40 species and Aβ42 propagates the Aβ42 isoform as suggested by some studies (Hasegawa et al. 1999; Kim et al. 2007; Yan & Wang 2007). However, in the case of the secondary transmission studies presented here, only Aβ42 was left as a seed to induce aggregation and was sufficient to recapitulate the strain-like morphologies. These results are consistent with various studies indicating that pre-formed fibrils of Aβ40 and Aβ42 can promote each other's aggregation (Gu & Guo 2013; Gu et al. 2016; Hasegawa et al. 1999; Jan et al. 2008; Jarrett et al. 1993; Ono et al. 2012; Snyder et al. 1994) and can share strain-like features. Additionally, this study ties in with previous results suggesting that the seeding activity of Aβ in transgenic mouse lines peaks during early phases of deposition that are characterized by a temporary sharp increase in the overall Aβ42/Aβ40 ratio (Ye et al. 2017).

Given the importance of the Aβ42 isoform in familiar forms of the disease and the fact that parenchymal senile plaques are almost entirely composed of the Aβ42 peptide, our results emphasize the importance

of the Aβ42 peptide in the pathophysiology of AD. In general, this study shows that fibril polymorphisms are very resistant to *in vivo* degradation and can serially be propagated. Plaque polymorphisms is not primarily caused be the underlying Aβ40/Aβ42 ratio but rather by distinct Aβ42 fibril morphologies that are very resistant to cellular degradation processes.

The previous study made use of focal injections into transgenic mouse hippocampi and provided insights into the durability of Aβ seeds at the point of injection. An intriguing feature of prion proteins involves the ability to propagate into distinct axonally connected areas causing specific spatiotemporal deposition pattern mirroring the characteristics of prion strains. A similar pattern of deposition has been suggested both for AD patients as well as depositing transgenic mouse models. In contrast to injections using α-synuclein fibrils and tau fibrils, the injection of Aβ aggregates into wildtype mice does not cause plaque deposition over prolonged incubation periods. As a consequence, current possibilities to study the propagation of Aβ seeds *in vivo* are restricted to transgenic mouse lines that develop endogenous plaques over time. Those endogenous plaques cannot be efficiently distinguished from the plaques induced by the exogenous seed. To understand the mechanisms that cause the spatial and temporal propagation of the Aβ protein, it is therefore reasonable to develop new mouse models that enable to follow the aggregation process *in vivo*.

2.4 The spatiotemporal pattern of protein seeding

2.4.1 Braak staging and propagation within axonally connected areas

Amyloidogenic diseases are characterized by the specific spatiotemporal aggregation of distinct proteins. In AD patients, both Aβ and tau show a progressive appearance of deposits over time. Initial neuropathological staging of AD was first described by Heiko Braak in 1991 and was specified by Thal in 2002 (Braak & Braak 1991; Thal et al. 2002). To clarify if there is a hierarchical involvement of Aβ plaque deposition in humans, the authors investigated the brains of clinically proven AD cases studying the amount of Aβ deposition in serial sections. Local differences regarding the frequency of Aβ deposition within specific brain regions allowed the categorization of five different phases. The first phase involves the deposition within the frontal, parietal, temporal and occipital neocortex whereas all other brain regions are free of amyloid deposits. In the second phase of deposition, plaques develop in allocortical areas as in the entorhinal cortex, CA1 region and insular cortex and subsequently deposit within subcortical regions including diencephalic nuclei and the striatum during the third phase. The next phase represents first plaque deposits within distinct brainstem nuclei (substantia nigra, red nucleus, central gray, superior and inferior collicle, inferior olivary nucleus, and intermediate reticular zone). In the final phase, the cerebellum and additional brainstem nuclei are increasingly involved (Thal et al. 2002). One implication from these studies is that first Aβ fibrils are formed within neocortical areas and propagate anterogradely into regions that are neuronally connected with regions that already exhibit Aβ deposition. Since different regions become involved in a temporally distinct pattern, regional susceptibility may likely play an additional role in the deposition process. In this case, the prion-like

self-seeding characteristics of the Aβ peptide give rise to a stereotypical aggravation of the disease over time. Additionally, nondemented cases with AD-like pathologies can occasionally exhibit Aβ deposition phases one to three. One major characteristic of AD is the long clinically silent phase that precedes the onset of clinical dementia. This phase has been suggested to begin 10 to even 20 years earlier (Bateman et al. 2012a; Holtzman et al. 2011; Selkoe 2011). Therefore, it is tempting to speculate that these nondemented cases represented preclinical stages of the disease.

The neuropathological spreading of neurofibrillary tangles and neuropil threads appears to be partially reversed to the spatiotemporal deposition of Aβ plaques and permits to be differentiated into six different stages. Both the first and second stage of NFT deposition take place in the entorhinal cortex and gradually expand to the limbic stages, identified as phase three and four, showing deposition in parts of the hippocampus. Within the last two stages of NFT deposition, nearly all isocortical association areas are involved (Braak & Braak 1991). According to evidence that NFT correlate better with cognitive impairment than Aβ plaques (Dickson et al. 1992), the entorhinal stages most probably correlate to clinically silent periods of the disease, whereas limbic and isocortical stages represent a gradual worsening of the dementia (Braak & Braak 1991) (**Figure 5**).

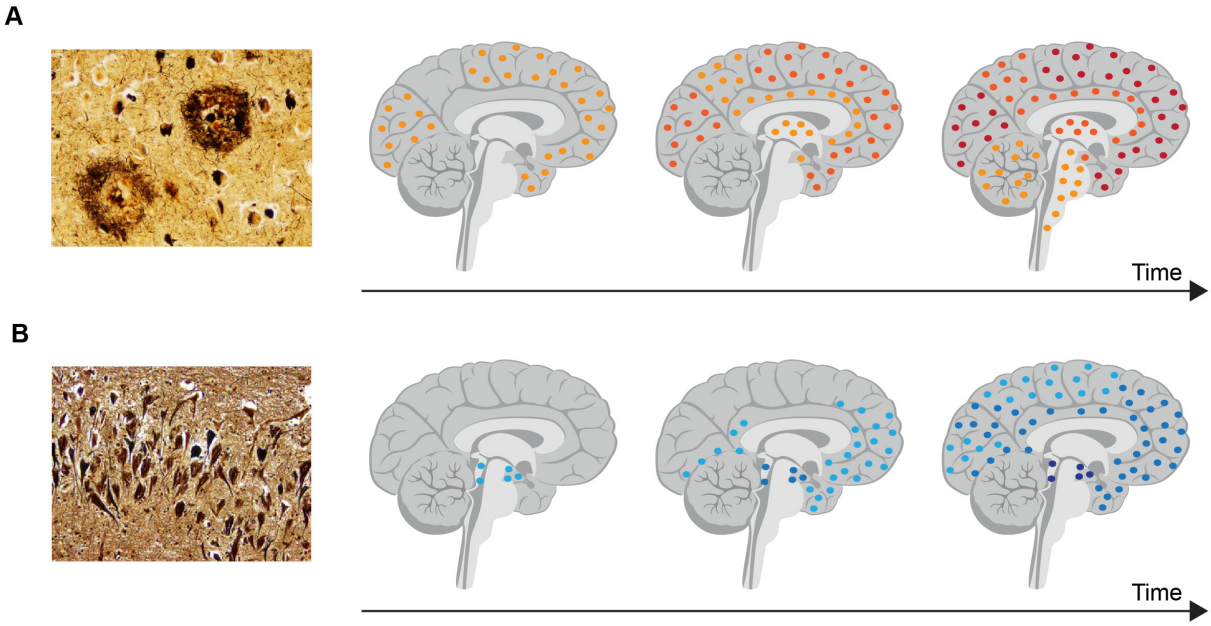

Figure 5. Progressive appearance of protein deposits in human AD patients. Specific proteinaceous lesions in neurodegenerative diseases show a characteristic progression as shown by post-mortem analyses of patient material. **A)** Aβ plaque deposition and **B)** tau inclusions in brains of patients with AD. Three stages of protein propagation are shown and illustrated by different colors.

In vivo studies in APP transgenic mice favor the neuropathological results of AD patient brain tissue. Young transgenic mice develop first deposits within neocortical areas followed by a spread of deposition to other brain regions while animals age (Irizarry et al. 1997; McGowan et al. 1999). In a similar manner, Aβ seeds that are intracranially injected into specific regions of the brain trigger deposition in axonally connected areas over time. These regions include neocortical and subcortical areas, similar to those areas characterized by Braak staging (Hamaguchi et al. 2012; Jucker & Walker 2011; Ye et al. 2015a). Although the mechanism of *in vivo* propagation to distinct parenchymal areas is not yet solved, in vitro studies give rise to the assumption that the seeding is mediated by neuronal uptake, transport and subsequent release of soluble oligomers into the extracellular environment to form new fibrils (Domert et al. 2014; Nath et al. 2012). Domert et al. could show that particularly the $A\beta_{42}$ isoform promoted cell-to cell transfer in a fast fashion, most likely due to its tendency to aggregate which makes it more resistant to degradation (Ahmed et al. 2010; Domert et al. 2014). Additionally, extracellular oligomers can be spontaneously internalized by neurons into endosomes and lysosomes (Nath et al. 2012). Over time, the intraneuronal accumulation of Aβ oligomers results in neuronal stress (Lee et al. 2011). These results implicate that Aβ seeds translocate within the brain already very early during the disease – likely as a stress response to get rid of the aggregated peptide and well before first neurons start to deteriorate. These findings demonstrate the extensive mobility of Aβ within the central nervous system. The suggestion that soluble Aβ seeds spread already very early during the disease within different brain areas has important implications for the pathomechanism of the disease and can be roughly compared to metastasis in various forms of cancer (Jucker & Walker 2018).

2.4.2 New transgenic mouse models to study the propagation of Aβ seeds in vivo

In reference to:
Beschorner N, Ruiz-Riquelme AI, Uhlmann RE, Werner R, Häsler LM, Kaeser SA, Stauffenbiel M, Walker L and Jucker M. Induction of murine cerebral β-amyloidosis as a tool to study prion-like propagation within the brain. in preparation

Unfortunately, it is very difficult to follow seeding mechanisms in *in vivo* models due to the lack of differentiation between the initial seeds and the following aggregation process per se. On the other hand, *in vitro* seeding models are not able to recapitulate the complete process of aggregation although providing strong evidence for the validity of the prion paradigm. One of the main obstacles that impede the study of *in vivo* seeding mechanisms is the fact that synthetically or recombinantly designed Aβ that could be easily distinguished by fluorescent tags or differences in amino acid sequence do not efficiently lead to plaque deposition in transgenic animals (Meyer-Luehmann et al. 2006). To overcome this hurdle, we made use of the fact that endogenous murine Aβ differs from its human homologue in three amino acids at residues 5, 10 and 13 leading to a change of the amino acids arginine to glycine, tyrosine to

phenylalanine and histidine to arginine (Yamada et al. 1987). The two forms of the protein can be distinguished by commercially available antibodies which makes it possible to observe the co-aggregation of both proteins.

In comparison to human Aβ, the probability of murine Aβ to aggregate seems to be limited and wild-type mice do not develop Aβ plaques spontaneously (Xu et al. 2015). Interestingly, a recent study analyzing aged wild-type mice concluded that Aβ gradually increases in the cytosol and lysosomes of cortical neurons as well as extracellularly deposits in the periventricular zone(Ahlemeyer et al. 2018). Additional data demonstrate the ability of murine Aβ to aggregate: *In vitro* studies report on mixed amyloid fibrils consisting of murine and human Aβ peptide (Fung et al. 2004). In human APP transgenic mice, murine endogenous Aβ is usually expressed in combination with the overexpressed human transgene and both isoforms co-deposit in senile plaques (Mahler et al. 2015; Morales-Corraliza et al. 2013; Pype et al. 2003; van Groen et al. 2006). However, when wild-type mice are injected with APP transgenic material, they do not demonstrate seeded Aβ deposition (Kane et al. 2000; Meyer-Luehmann et al. 2006).

For further evaluation of the aggregation characteristics of human and murine Aβ peptide, we generated three new mouse models: *APP_{swe}-GFR*, *APP_{swe}-GFR x PS1 G384A* and *APP23 x APP-knockout*. The *APP_{swe}-GFR* and *APP_{swe}-GFR x PS1 G384A* mouse lines both express murine Aβ using the Thy1 promotor with a three-fold overexpression. The *APP_{swe}-GFR x PS1 G384A* mouse line additionally expresses the PS1 mutation G384A. Whereas the *APP_{swe}-GFR* mouse strain does not deposit Aβ plaques during its normal lifespan, the *APP_{swe}-GFR x PS1 G384A* mouse strain starts to deposit at around 10 months of age including parenchymal diffuse deposits as well as CAA. *APP_{swe}-GFR* mice demonstrate a high $A\beta_{40}/A\beta_{42}$ ratio. On the contrary, the addition of the *PS1 G384A* mutation causes a shift of the $A\beta_{40}/A\beta_{42}$ ratio that likely represents the trigger for the deposition of murine Aβ. Interestingly, these findings support the results described in the chapter above revealing the importance of the $A\beta_{42}$ isoform as the primary proteinaceous seed in AD. The *APP23 x APP-knockout* mouse line represents the APP23 mouse strain that was already described in detail earlier and crossbred to the APP-knockout mouse line that is deficient of APP. As a result, the *APP23 x APP-knockout* mouse line does not express endogenous murine Aβ but only a six-fold overexpression of human Aβ. Mahler et al. characterized the *APP23 x APP-knockout* mouse model and could show that it comprises a 35% decreased cerebral Aβ load compared to aged-matched APP23-mice demonstrating that murine and human Aβ peptides intercalate during deposition (Mahler et al. 2015). In comparison to amyloid deposits that are either entirely created of human Aβ or a mixture of human transgene and endogenous murine Aβ that appear compact and Congo Red positive, amyloid deposits made up of murine Aβ have a rather diffuse morphology and are rarely Congo Red positive. LCO analysis using the fluorescent dyes hFTAA and qFTAA support the morphological differences observed by histology and may indicate conformational polymorphism. The results are in comparison with a study that reported an increased solubility of amyloid deposits when

murine Aβ was overexpressed simultaneously with human Aβ in APP transgenic mice (Jankowsky et al. 2007).

Human Aβ injected intracranially into wild-type mice does not cause plaque deposition over extended incubation periods. To study if murine Aβ in the non-depositing *APP_{swe}-GFR* mouse line can be induced by a prion-like mechanism, human *APP23 x APP-knockout* brain homogenate as well as murine *APP_{swe}-GFR x PS1 G384A* brain homogenate was intracranially injected. Twelve months post injection, both extracts induced robust seeding in the *APP_{swe}-GFR* host and strain-like morphologies of the injected material could be partially conveyed by seeded transmission. Structural polymorphisms manifested in histological plaque appearance and distinct emitted LCO spectra. In both cases, the depositing plaque material was entirely made of murine Aβ, and therefore originated from the host environment. This was confirmed by histological stainings as well as ultra-sensitive bead-based single-molecule array Simoa technology. The results manifest the implication of the prion paradigm for the seeded aggregation of the Aβ protein *in vivo*: In the presence of an adequate seed, in this case either human or murine brain homogenate, the templated misfolding of soluble endogenous protein, in this case murine Aβ, is induced and deposition becomes histologically observable after a lag phase of a variable time span (Beschorner et al., in preparation).

Furthermore, the injected brain homogenate does not only cause Aβ depositions in the focally injected area, the hippocampus, but propagates to axonally connected areas over time. In case of the *APP_{swe}-GFR* mouse strain, these brain areas include the entorhinal cortex, the frontal cortex and olfactory bulb, the fornix, the mammillary bodies, extensive CAA in the meninges as well as parts of the parietal and temporal cortex above the injection site. Regarding the temporal pattern of plaque deposition, strong deposition of CAA in the meninges appears six months post injection, followed by hippocampal plaque deposition around 8 months post injection. After 10 months, the entorhinal cortex becomes increasingly involved and at round 12 months post injection, Aβ deposition becomes visible in the anterior olfactory bulb and frontal cortex. Similar to earlier studies, the predominant neuroanatomic pattern of emerging Aβ deposition seems to follow the neuronal connectivity of the limbic system (Ye et al. 2015a). Interestingly, the anterior thalamus, which is part of the classical Papez circuit (entorhinal cortex, anterior thalamus and mammillary bodies) does not exhibit plaque aggregation which is in contrast to previous studies (Ronnback et al. 2012; Ye et al. 2015a). The organization of the hippocampus is complex and not yet fully understood. Recent results redefine the hippocampus' multiscale network organization and elucidate the complicated retrograde and anterograde brain-wide connectivity patterns (Bienkowski et al. 2018). The different anatomical parts within the hippocampus are connected via intrahippocampal projections that may explain the strong Aβ depositions within the entire hippocampus after a focal injection into the dentate gyrus. Intrahippocampal injections additionally involve anterograde as well as retrograde connections to the entorhinal cortex (Cenquizca & Swanson 2007). Other axonal connections emerge from the fornix to innervate the anterior olfactory nucleus and parts

of the prefrontal cortex. These connections are mainly considered to be of an anterograde origin (Aqrabawi & Kim 2018; Bienkowski et al. 2018). In the case of deposition in the temporal and parietal neocortex on top of the injection sites, it cannot be excluded that a small part of the injected seeds leaked into the brain parenchyma along the injection track. The extensive and early involvement of CAA in the meninges is most likely explained by injury to blood vessels during the injection process. These findings reveal that Aβ seeds propagate to neuronally connected areas by either retrograde or anterograde transport similar to observed stages of Aβ deposition in the Braak staging of AD. Due to the complex connectivity of the hippocampal structures, it is still unclear if rather anterograde, retrograde or bilateral neuronal connections involve the propagation of the Aβ seeds. However, the results presented here argue for a bilateral propagation of Aβ seeds within axonally connected areas. These results are encouraged by a plethora of recently published articles emphasizing the relevance of both anterograde and retrograde axonal connection in the spreading of α-synuclein fibrils (Mezias et al 2020), tau accumulation in Alzheimer's disease (Franzmeier et al., 2020) as well as the propagation of $A\beta_{42}$ peptides in primary cortical neurons grown in microfluidic devices (Brahic et al., 2016). The latter study additionally implicates that anterograde and retrograde transport of $A\beta_{42}$ occurs to the same amount and can be secreted into the neuronal surrounding without axonal break-down, indicating that trans-neuronal spread can occur in intact healthy neurons. In addition to an exclusive involvement of axonally connected areas in the propagation of protein deposits, it might be that a specific neuronal vulnerability gives rise to the observed propagation pattern. In this case, aggregated proteins may spread to many neighboring and axonally connected regions, but only the vulnerable regions will eventually give rise to protein deposits over time (Freer et al 2016).

The results of a spreading of Aβ deposition within the limbic connectome are in line with the observation that AD patients suffer from a general neuronal network connectivity breakdown (Daianu et al. 2013; Filippi et al. 2010). Another publication demonstrated the specific decreased functional connectivity of the hippocampus with the cerebral cortex and limbic areas (Allen et al. 2007). Growing evidence therefore implicates the breakdown of the neuronal network as causative for some of the symptoms as the disease progresses and provides a correlation between clinical symptoms of AD patients and the possible prion-like propagation of the Aβ seed (Pascoal et al. 2019).

2.5 Conclusion & Outlook

Within the last decades, the amyloid cascade hypothesis has been the seminal influence on research and treatment strategies for AD (Hardy & Selkoe 2002; Strooper & Karran 2016). The insight that the disease belongs to a specific group of neurodegenerative disorders that are caused by the aggregation of particular proteins in a prion-like fashion have shed a completely new light on the onset and progression of AD. Within this dissertation, it has been shown that the Aβ protein is able to aggregate into distinct conformational variants in AD patients. Plaques morphologies can be used to discriminate between familiar and sporadic forms of the disease and considerable variety even appeared within the sporadic patient group (Rasmussen et al. 2017). The differences in Aβ conformations could be propagated to susceptible transgenic mouse strains, an important feature of strain-like variability (Aguzzi et al. 2007). An important insight of the study was the finding that even within a single AD brain, not just one dominant plaque conformation exists but a variety of different polymorphisms that cluster into clouds of conformations.

The AD brains analyzed in this study all displayed end stage pathologies of the disease. Some studies imply that Aβ morphologies may change over the course of the disease and therefore the morphological variety observed could vary from the conformation of the earliest Aβ seeds (McDade & Bateman 2017; Ye et al. 2017). Symptomatic AD is the result of a long preclinical phase involving protein aggregation and subsequent neuronal loss. Recent results even suggest that first protein deposits appear up to 20 years before first symptoms of cognitive decline (Bateman et al. 2012b). In terms of therapeutic options, it is important to understand the progressive mechanisms that cause the pathophysiology of AD and if the Aβ deposits observed in end stage brains can shed light on the earliest aggregation mechanisms of the disease.

In the subsequent study, APP transgenic mice were therefore used to analyze the conformational stability of distinctive Aβ aggregates and the nature of the earliest seeds giving rise to disease onset. It was conclusively shown that strain-like conformations of different Aβ aggregates are very resistant to degradation in an *in vivo* environment and can be propagated over multiple passages. This might implicate that morphological differences in end stage AD brain are similar to the earliest Aβ aggregates and do not change significantly over the course of the disease process. Furthermore, the data suggest that the $A\beta_{42}$ peptide is likely the most important Aβ isoform in the whole seeding process and responsible for various plaque polymorphisms *in vivo* (Beschorner et al., in preparation). These results go along with the early finding that plaque cores of AD patients almost entirely consist of the $A\beta_{42}$ isoform. This study and the one mentioned above emphasize that Aβ aggregate conformations in an *in vivo* environment are likely to show striking discrepancies among patient subgroups as well as within a single AD patient. However, the conformation of a single plaque could be very consistent and stable

during the course of the disease. One explanation for the change in plaque morphologies during ageing as described in some transgenic mouse models (Ye et al. 2017) could therefore be a conformational selection and preferential seeding of more dominant strains over time-

Another intriguing feature of the prion protein involves the ability to propagate into distinct axonally connected areas causing specific spatiotemporal deposition pattern mirroring the characteristics of prion strains. A similar pattern of deposition has been suggested both for AD patients as well as depositing transgenic mouse models. In the third study, the development of either entirely murine or human mouse models was used to develop an *in vivo* method to study the propagation of Aβ deposition over time. Preliminary results hint towards an implication of both anterograde as well as retrograde transport regarding the spreading of Aβ seeds (Beschorner et al., in preparation). *In vivo* models to study the molecular mechanisms of amyloid aggregation and subsequent spreading within the brain are currently missing. Therefore, this newly developed tool to characterize the aggregation process might provide important insight into the spatiotemporal deposition and observed regional vulnerability. Injection of brain homogenate of the *APP23 x APP-knockout* mouse line (comprising human Aβ) into the non-depositing APP_{swe}*-GFR* mouse line (comprising mouse Aβ) can be used to follow the propagation of the human seed in the murine environment. Human and mouse Aβ can be distinguished with commercially available antibodies and measured with ultra-sensitive bead-based single-molecule array Simoa technology. This tool will provide important insights on how Aβ seeds interact with monomeric endogenous Aβ peptides and how the following propagation to distinct brain regions takes place. Additionally, these mouse models can be used to study the effects of regional vulnerability on protein deposition in AD. To find out if Aβ seeds propagate to more regions than those that exhibit Aβ lesions, reinjection of non-depositing dissected brain areas into susceptible transgenic mice will provide important new information.

Considering the results of this doctoral thesis as a whole, it is a strong proof of the similarities between prions and the Aβ peptide. The first study provides strong evidence that Aβ plaque cores display considerable heterogeneity in postmortem AD tissue and the second and third study demonstrate both the long-lasting persistence as well as the *in vivo* propagation of the Aβ protein in a prion-like seeding mechanism. Prion strains are known to correlate with different clinical symptoms of the disease (Solforosi et al. 2013). Whether different Aβ plaque conformations give rise to specific clinical symptoms or alter the course of the disease is not yet known. Especially in the group of sporadic AD cases, a significant fibril heterogeneity between patients was discovered. For the future, it is essential to analyze the plaque morphologies in larger groups of sporadic patients to investigate a potential correlation to clinical heterogeneity. As far as the translational perspective of this study is concerned, these data might provide insight into the recent concerns regarding the efficiency of anti-Aβ immunotherapies (Condello et al. 2018). A high conformational variability both among patients as well

as within a single AD brain can indeed have an impact on the efficiency of anti-Aβ immunotherapy. In this regard, a combination of different Aβ antibodies might possibly provide more encouraging results.

Currently, most treatment approaches for AD are moving towards primary prevention studies. Therefore, biomarkers for the detection of early Aβ peptide deposition are essential. PET scanning with the ligand Pittbourgh Compound B (PiB) is used to assess the disease status of suspected AD patients. The retention of the PET ligand, PiB or other compounds that bind to the amyloid-conformation of Aβ plaques within the brain, is measured (Clark et al. 2012; Klunk et al. 2004; Wolk et al. 2012). However, the presence of different plaque conformations may falsify the results of PET imaging as demonstrated by the unique case of a patient with sporadic AD being negative for PiB binding (Rosen et al. 2010). As a consequence, it is essential to investigate the structural features of the Aβ peptide during the earliest stages of pathophysiology (McDade & Bateman 2017, Beschorner et al. in preparation).

Since the publication of the amyloid cascade hypothesis in 1992, the Aβ peptide has been considered the central player in AD pathology (Hardy & Allsop 1991). The discovery that prions and the Aβ protein share similar characteristics shifted the focus on the pathophysiology that gives rise to protein misfolding and the resulting neurodegeneration (Jucker & Walker 2013). The studies presented in this dissertation have provided further evidence for the similarities between prions and the Aβ peptide. Aβ can aggregate into distinct conformations that specifically depend on the disease status (Rasmussen et al. 2017) and the highly amyloidogenic isoform $A\beta_{42}$ is extremely resistant towards cellular degradation mechanisms and gives rise to strain-like conformations despite of differences in $A\beta_{42}/A\beta_{40}$ ratios (Beschorner et al. in preparation). Lastly, a powerful tool was established to follow the aggregation and propagation of Aβ seeds within an *in vivo* mouse model. These findings will significantly help to understand the neurotoxic effect of Aβ aggregation in AD and provide new tools for further insights into this disease.

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3. Publications

3.1 Description of personal contribution

I. Amyloid polymorphisms constitute distinct clouds of conformational variants in different etiological subtypes of Alzheimer's disease.

Rasmussen J*, Mahler J*, **Beschorner N***, Kaeser SA, Häsler LM; Baumann F, Nyström S, Portelius E, Blennow K, Lashley T, Fox NC, Sepulveda-Falla D, Glatzel M, Oblak AL, Ghetti B, Nilsson KPR, Hammarström P, Staufenbiel M, Walker LC, Jucker M.

*equal contribution

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Personal Contribution: Experimental design and planning of the study (together with JR, JM, KPRN, PH, MS, LCW and MJ); Mouse injections, histology and stereology (together with JR); Data analysis and statistical analysis (together with JR and JM); Figure design and preparation (together with JR); Writing of the manuscript (together with all the other authors).

Others: JR, JM, LMH, EP, TL, DSF and ALO performed the experimental work. JR, JM, SAK, LMH, FB, SN, EP, KB, TL, NCF, DSF, MG and BG carried out the analysis. JR, JM, KPRN, PH, MS, LCW and MJ designed the experiments and wrote the manuscript with help from all other authors.

II. Persistence of amyloid-β plaque polymorphism in APP null mouse brain is mediated by the $A\beta_{42}$ isoform

Beschorner N, Ye L, Häsler LM, Jucker M

in preparation

Personal contribution: Experimental design and planning of the study (together with LY and MJ); Mouse injections and histology (together with LY); Homogenization of tissue and SIMOA measurement (together with LMH), Data analysis and statistical analysis (together with LMH and MJ); Figure design and preparation; Writing manuscript (together with MJ).

Others: YL and LMH performed the experimental work. LMH carried out the analysis. LY and MJ designed the experiments.

III. Induction of murine cerebral β-amyloidosis as a tool to study prion-like propagation within the brain

Beschorner N, Ruiz-Riquelme AI, Uhlmann R, Häsler LM, Werner R, Kaeser SA, Staufenbiel M, Walker LC, Jucker M.

In preparation

Personal contribution: Experimental design and planning of the study (together with AIRR, MS, LCW and MJ); Mouse injections and histology; Homogenization of tissue and SIMOA measurement (together with AIRR, RU and LMH), Data analysis and statistical analysis (together with AIRR and MJ); Figure design and preparation; Writing manuscript (together with AIRR, MS, LCW and MJ).

Others: AIRR, RU and LMH performed the experimental work. AIRR, RU and LMH carried out the analysis. AIRR, MS, LCW and MJ designed the experiments.

3.2 Amyloid polymorphisms constitute distinct clouds of conformational variants in different etiological subtypes of Alzheimer's disease

Rasmussen J*, Mahler J*, Beschorner N*, Kaeser SA, Häsler LM, Baumann F, Nyström S, Portelius E, Blennow K, Lashley T, Fox NC, Sepulveda-Falla D, Glatzel M, Oblak AL, Ghetti B, Nilsson KPR, Hammarström P, Staufenbiel M, Walker LC, Jucker M.

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*equal contribution

3.3 Persistence of amyloid-β conformers in APP null mouse brain is mediated by the $A\beta_{42}$ isoform

Beschorner N, Ye L, Häsler LM, Jucker M
In preparation

Persistence of Aβ conformers in APP null mouse brain is mediated by the $A\beta_{42}$ isoform

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Abstract

Cerebral β-amyloidosis can be induced by intracerebral injections of brain extract containing aggregated forms of β-amyloid (Aβ seeds) into susceptible transgenic mice. Previous work could show that the induction of protein deposition is caused by a seeding mechanism that involves the templated misfolding of endogenous Aβ. The β-amyloidosis-inducing factor likely consists of Aβ assemblies, ranging from small soluble Aβ oligomers to larger fibrils. Here we report the persistence of Aβ confomers upon inoculation in *App*-null mice for up to 6 months with secondary transmission in APP transgenic mice. Using ultra-sensitive immunoassay, we could show that the Aβ-inducing activity as well as the signatures of polymorphism in APP transgenic mice is mainly caused by the Aβ42 isoform and not due to differences in Aβ42/Aβ40 ratios. The resistance of the Aβ42 isoform to inactivation and structural modification by cellular degradation mechanisms underscores its implication in disease onset and raises the possibility that Aβ42 mediates the spread of Aβ deposition within the brain.

Introduction

Protein misfolding can give rise to a pathogenic process in which specific proteins progressively aggregate into intracellular or extracellular deposits resulting in the onset of various neurodegenerative diseases. In the case of Alzheimer's disease (AD), the aggregation of the β-amyloid (Aβ) protein induces parenchymal deposits as well as cerebral amyloid angiopathy (CAA). Recent data suggest that the progressive protein aggregation observed in many neurodegenerative diseases is reminiscent of the endogenous misfolding, aggregation and subsequent spreading of prions culminating in fatal neurodegeneration. In analogy, prion-like propagation of the Aβ protein involves the misfolding into proteopathic seeds that structurally corrupt endogenous Aβ peptide further aggravating the seeding cascade (Jucker & Walker 2013).

The intracerebral injection of Aβ-laden brain extract from either AD brains or aged APP-transgenic mouse brains is able to induce Aβ deposition in the brain parenchyma of young predepositing APP-transgenic mice(Kane et al. 2000; Meyer-Luehmann et al. 2006; Watts et al. 2014; Ye et al. 2015). The seed that induces the aggregation of the Aβ protein has been identified as a variety of aggregated Aβ forms, ranging from small soluble Aβ oligomers to larger fibrils (Langer et al. 2011). The two most abundant Aβ isoforms in the brain are Aβ40 and Aβ42, with the latter being more hydrophobic and therefore displaying a higher potential to aggregate (Haass & Selkoe 2007; Karran et al. 2011). Recently, polymorphic aggregates observed in the APP transgenic mouse models APP23 and APPPS1 have been partly ascribed to the differences in their Aβ42/Aβ40 ratios (Heilbronner et al. 2013a). Aβ aggregate variations have also been described in post-mortem AD brains and a possible correlation to clinical heterogeneity is currently under debate (Rasmussen et al. 2017). Thus, the aim of the present study was to test the resistance of strain-like polymorphisms towards *in vivo* degradation and the influence of different Aβ42/Aβ40 ratios on aggregate structures as well as seeding efficiency.

Methods

Mice

For all experiments described here, either aged (>20 months old) or young (2-3months old) female APP23, APPPS1 and APP-knockout mice were used. APP23 mice express KM670/671NL mutated APP under control of the neuronal Thy1 promotor. The mice have been backcrossed with C57BL/6J mice for more than 25 generations (C57BL/6J-Tg(Thy1-$APP_{K670N;M671L}$)23). APP23 mice are currently bred at the Hertie Institute for Clinical Brain Research. Female mice develop first Aβ deposits in the neocortex at 7 months of age and in the hippocampus between 8-9 months of age (Sturchler-Pierrat et al. 1997). APPPS1 mice express KM670/671NL mutated APP under control of the neuronal Thy1 promotor as well as L166P mutated presenilin 1 and were generated and maintained on a C57BL/6J genetic background at the Hertie Institute for Clinical Brain Research. Female APPPS1 mice develop first neocortical Aβ deposits at around 2 months of age (Radde et al. 2006). All mice were group-housed under pathogen-free conditions. All experimental procedures performed were undertaken in accordance with the veterinary office regulations of Baden-Württemberg (Germany) and approved by the local Animal Care and Use Committees.

Preparation of brain tissue extracts

Brain extracts for primary injections were derived from aged depositing female APP23 transgenic mice (28-30 months old) and aged depositing female APPPS1 transgenic mice (20-22 months old). After removal of the cerebellum and lower brainstem, the forebrain was immediately fresh-frozen on dry ice and stored at -80°C until use. Tissue was then homogenized at 10% (w/v) in sterile phosphate-buffered saline (PBS, Lonza, Switzerland) using the Precelly system with a 2 × 20-s cycle at 5,500 rpm

(Precellys24 Homogenizer, EQ03119- 200-RD000.0, Bertin Instruments). Afterwards, samples were vortexed and centrifuged at 3,000 x g for 5 minutes as previously described (Eisele et al. 2010; Meyer-Luehmann et al. 2006). The supernatant was aliquoted and immediately frozen. For all experiments, the 10% (w/v) extract was used. Brain extracts for secondary injections were derived from the dissected hippocampi from injected APP-knockout mice. The preparation of the extracts was performed as described above. Extracts from the primary inoculation in each group were pooled for secondary inoculation.

Stereotactic injection of brain extracts

APP23 and APP-knockout mice were anaesthetized with a mixture of ketamine (100mg/kg body weight) and xylazine (10mg/kg body weight) in saline. Bilateral stereotactic injections of 2.5µl brain extract were performed with a Hamilton syringe that was placed into the hippocampus (AP - 2.5mm, L +/- 2.0mm. DV -1.8mm). Injection speed correlated to 1.0µl/minute and the needle was kept in place for additional 2 minutes before slowly withdrawn from the brain parenchyma. The surgical area was cleaned with sterile saline, the incision was sutured and the mice were monitored until recovery from anesthesia and within the following weeks.

Histology and immunohistochemistry

Before staining, brains were removed and placed in 4% paraformaldehyde in PBS for approximately 48 hours for immersion-fixation. Afterwards, brains were cryoprotected in 30% sucrose in PBS for additional 48 hours. Brains were frozen in 2-methylbutane cooled with dry ice and afterwards serially cut into 25 µm-thick coronal sections with the use of a freezing-sliding microtome. Collected tissue was placed in cryoprotectant (35% ethylene glycol, 25% glycerol in PBS) and stored at -20 °C until use. For immunostaining of Aβ, the polyclonal antibody CN6 was used. Sections were counterstained with Congo Red according to standard protocols.

Histological staining with hFTAA/qFTAA and spectral analysis

For spectral analysis, coronal sections of inoculated mice were stained with a combination of the LCO dyes hFTAA and qFTAA. 25 µm-thick coronal sections were washed in PBS (3x 10 min) and mounted on Superfrost slides. Sections were subsequently dried for 2 hours at room temperature. Staining with both LCOs was performed as previously described (2.4 µM qFTAA and 0.77 µM hFTAA in PBS). Sections were incubated for 30 min at RT in the dark. Spectra were acquired on a Zeiss LSM 510 META confocal microscope equipped with an argon 458-nm laser for excitation and a spectral detector (Carl Zeiss MicroImaging GmbH). A 40× oil-immersion objective (1.3 N.A.; Zeiss) was used for spectral imaging of Aβ-amyloid cores. Continuous emission spectra were acquired from 470 to 695 nm. The amyloid plaques were randomly chosen, and three regions of interest

were mea- sured in the core of each deposit. Analysis was restricted to the cores of plaques. At least 20 Aβ plaque cores were measured in each mouse. For analysis, all emission spectra were normalized to their maxima and the mean spectral signature of each plaque core was calculated before averaging the values for each mouse. The ratio of the intensity of emitted light at the blue-shifted peak (502 nm) and red-shifted peak (588 nm) was used as a parameter for spectral distinction of different Aβ deposits. The peaks of the spectra were selected to maximize the spectral distinction.

Quantification of Aβ by immunoassays

For quantification of Aβ concentration, extracts were pretreated with formic acid (Sigma-Aldrich, final concentration: 70% vol/vol), sonicated for 30s on ice and centrifuged at 25,000g for 1 hour at 4°C. Supernatants were neutralized in neutralization buffer (1M Tris base, 0.5M Na_2HPO_4, 0,05% NaN_3 (wt/vol)). Human Aβ was measured either by an electrochemiluminescence (ECL)-linked immunoassay (Meso Sclae Discovery, MSD), as already described or by an ultra-sensitive bead-based single molecule array (Simoa, Quanterix). In each case, samples and calibrators were run in duplicates.

Regarding the measurement on the MSD platform, a commercial Human (6E10) Abeta Triplex Assay was used according to the manufacturer's instructions. A 96-well plate pre-incubated with C-terminal capture antibodies against Aβx-38, Aβx-40 and Aβx-42 were blocked for 1 h with 1% bovine serum albumin (BSA in Tris buffer, wt/vol) and washed three times with Tris buffer. Formic acid–treated samples were co-incubated with the SULFO-TAG 6E10 detection antibody solution on the plate for 2 h. After washing, MSD Read Buffer T was added and the plate was measured immediately on the Sector Imager 6000. Data analysis used MSD DISCOVERY WORKBENCH software 2.0. Internal QC samples were used for quality control of the assay performance and inter-plate variability. Total Aβ was the sum of Aβx-38, Aβx-40 and Aβx-42.

In case of low Aβ levels, samples were measured using the commercial Simoa Human $Aβ_{40}$ and Human $Aβ_{42}$ kits from Quanterix. Depending on the expected Aβ concentration formic acid treated samples were diluted in sample diluent before being subjected to the fully automated analysis. Aβ was captured by antibody-coated beads and co-incubated with biotinylated detector antibodies against $Aβ_{40}$ (or $Aβ_{42}$) to form a complex. After a wash step, a streptavidin-β-galactosidase (SBG) conjugate was added and after further washing, resuspension in a substrate solution led to a fluorescence signal in the presence of SBG. Following transfer to the array disc, beads were individually sealed in microwells and quantified by the optical system of the Simoa HD-1 Analyzer. Each labeled Aβ molecule yielded a measurable signal. For the calibration, the software uses a Cubic ($1/Y^2$ with zero-point custom weight of 0.1) or a 4 Parameter Logistic ($1/Y^2$ weighted) curve fit data reduction method for the human $Aβ_{40}$ and $Aβ_{42}$ assays, respectively. For internal quality control, 3 in-house QC samples (at high, intermediate, and low Aβ concentration) were measured in each individual run. Total Aβ was the sum of $Aβ_{40}$ and $Aβ_{42}$.

Statistical analysis

Data in figures represent mean +/- s.e.m. Statistical analysis was performed using the GraphPad Prism software, version 6.0.

Results

The Aβ42 peptide is more resistant to in vivo degradation mechanisms

To study the nature and durability of the polymorphic Aβ deposits observed in the APP23 and APPPS1 mouse model, pooled brain homogenates from either aged APP23 or aged APPPS1 mice was inoculated into *App*-null mice. The *App*-null mouse line does not express APP and thus no Aβ peptide is generated. As a consequence, the injected brain extract cannot replicate by conformational conversion of endogenous Aβ peptide but is subject to *in vivo* degradation in the brain parenchyma. At 1, 7, 30, 60 and 180 days post inoculation (dpi) into the hippocampus, the injected hippocampi were dissected and residual Aβ concentrations were measured by immunoassay (Figure 1). At 1 dpi, the injected human Aβ peptide from both brain extracts could be detected in the hippocampi at similar levels. Human Aβ concentrations decreased over time and were below level of detection at 180dpi.

We previously found that the APP23 and APPPS1 mouse models differ regarding their Aβ plaque morphologies, spectral plaque properties as well as their respective Aβ42/Aβ40 ratios. The injected brain extracts were not normalized to equal Aβ concentration since differences in concentration may be a characteristic of the specific fibril polymorphism. The Aβ concentration of the injected APP23 extract was therefore almost 5 times higher than the injected Aβ concentration of the APPPS1 extract. Furthermore, the pool of APP23 extract comprised a strikingly higher Aβ40/Aβ42 ratio than the APPPS1 extract (Figure 1). Recently, these differences in Aβ isoform concentrations have been hypothesized to give rise to the observed plaque polymorphisms. The injected brain extracts displayed striking differences regarding their *in vivo* stability. In general, homogenate from APPPS1 mice appears to be more resistant towards cellular degradation than homogenate from APP23 mice. Interestingly, in both extracts, the Aβ40 isoform of the peptide dropped below level of detection within the first 30 dpi, whereas the Aβ42 isoform was detectable for more than 60 days in the APP23 extract as well as partly up to 180 days in the APPPS1 extract. These results are indicative of a higher *in vivo* stability of the Aβ42 protein.

Amyloid polymorphisms are mediated by the Aβ42 peptide and can be propagated via serial transmission

To assess the seeding activity of hippocampal extracts from *App*-null mice that had been injected with Aβ seeds from the polymorphic APP23 and APPPS1 mouse lines, secondary transmission studies into young predepositing APP23 host mice were performed (Figure 2). Therefore, the hippocampal APP-knockout extracts with incubation times of 1dpi, 7dpi, 30dpi, 60dpi and 180dpi were reinjected.

After an 8-month incubation period, the possibility of the residual Aβ seeds to induce conformational characteristics, as seen in the APP23 and APPPS1 strains, was assessed in terms of morphology, spectral differences and Aβ42/Aβ40 ratios.

Remarkably, primary incubation periods in the APP-knockout donor mice of up to 180 days did not change the polymorphic plaque characteristics of the residual Aβ seeds. Upon secondary injection into susceptible transgenic APP23 mice, the specific plaque polymorphisms could be faithfully replicated (Figure 2). Differences in morphological plaque appearance were supported by differences in spectral binding properties. Importantly, injected APP23 mice did not parallel the Aβ42/Aβ40 ratios of the injected brain homogenates but reflected the typical ratios of "unseeded" APP23 mice. These results strikingly emphasize the resistance of Aβ fibril conformations towards cellular degradation mechanisms. The pronounced *in vivo* stability of the Aβ42 protein implicates that this isoform passes on the strain-like conformations upon serial transmission instead of specific Aβ42/Aβ40 ratios as previously suggested.

Discussion

Our results emphasize the marked resistance of Aβ seeding activity as well as fibril polymorphisms towards clearance mechanisms within the living brain. In terms of seeding activity, it still remains elusive which Aβ species in the brain is most important for protein propagation and studies hint towards a variety of different aggregation forms (Langer et al. 2011). Apart from the differences in aggregate species that emerge during the seeding process, the two most abundant Aβ species, Aβ40 and Aβ42, display different characteristics. With its two additional amino acids, the Aβ42, isoform is more hydrophobic which results in a higher aggregation probability (Bitan et al. 2002; Haass & Selkoe 2007; Luhrs et al. 2005; Walsh & Selkoe 2007). The strain-like differences in the APP23 and APPPS1 mouse models have been partly ascribed to the differences in Aβ40/Aβ42 ratios (Heilbronner et al. 2013b; Novotny et al. 2016). While the resilience of Aβ seeds within the brain has already been described in detail (Bateman et al. 2012; Ye et al. 2015), our observations point towards a resistance of plaque polymorphisms over long incubations periods.

Furthermore, we could show that the structural differences observed in the transgenic mouse models were mostly driven by the Aβ42 isoform. Earlier studies showed that the Aβ40/Aβ42 ratio is maintained by seeded transmission and concluded that it might be the dominant factor that gives rise to strain-like

morphologies (Heilbronner et al. 2013; Novotny et al. 2016). The discrepancy between those and the current results might be explained by a selection mechanism whereby Aβ isoforms preferentially seed the same isoform if available. In the case of only the Aβ42 seed being available, cross-seeding with the more abundant Aβ40 species can occur that adopts the aggregate conformation of the Aβ42 seed. These results are consistent with various studies indicating that pre-formed fibrils of Aβ40 and Aβ42 can promote each other's aggregation (Gu & Guo 2013; Gu et al. 2016; Hasegawa et al. 1999; Jan et al. 2008; Jarrett et al. 1993; Ono et al. 2012; Snyder et al. 1994) and can share strain-like features. Additionally, this study ties in with previous results suggesting that the seeding activity of Aβ in transgenic mouse lines peaks during early phases of deposition that are characterized by a temporary sharp increase in the overall Aβ42/Aβ40 ratio (Ye et al. 2017). Furthermore, our results coincide with the finding that the Aβ42 peptide is also the principal protein species that deposits in parenchymal senile plaques within the AD brain and that the relative ratio of Aβ40/Aβ42 is closely related to the age of disease onset as well as a higher neurotoxicity in familial AD (Duering et al. 2005; Selkoe 2001).

The discovery that variant forms of fibril polymorphism exist in postmortem human AD brain with possible implications for the clinical course of the disease raises the question if fibril structures change of the course of the disease or rather maintain their structures (Lu et al. 2013; Qiang et al. 2017; Rasmussen et al. 2017). The discovery that aggregate variants are resistant towards in vivo aggregation imply that the morphological differences in end stage AD brain may be similar to the earliest Aβ aggregates that start the pathological processes within the brain. If strain-like fibril polymorphism is already present during the earliest phases of protein aggregation, the application of a combination of different Aβ antibodies might possibly provide more encouraging results that the current use of monoclonal anti-Aβ therapies.

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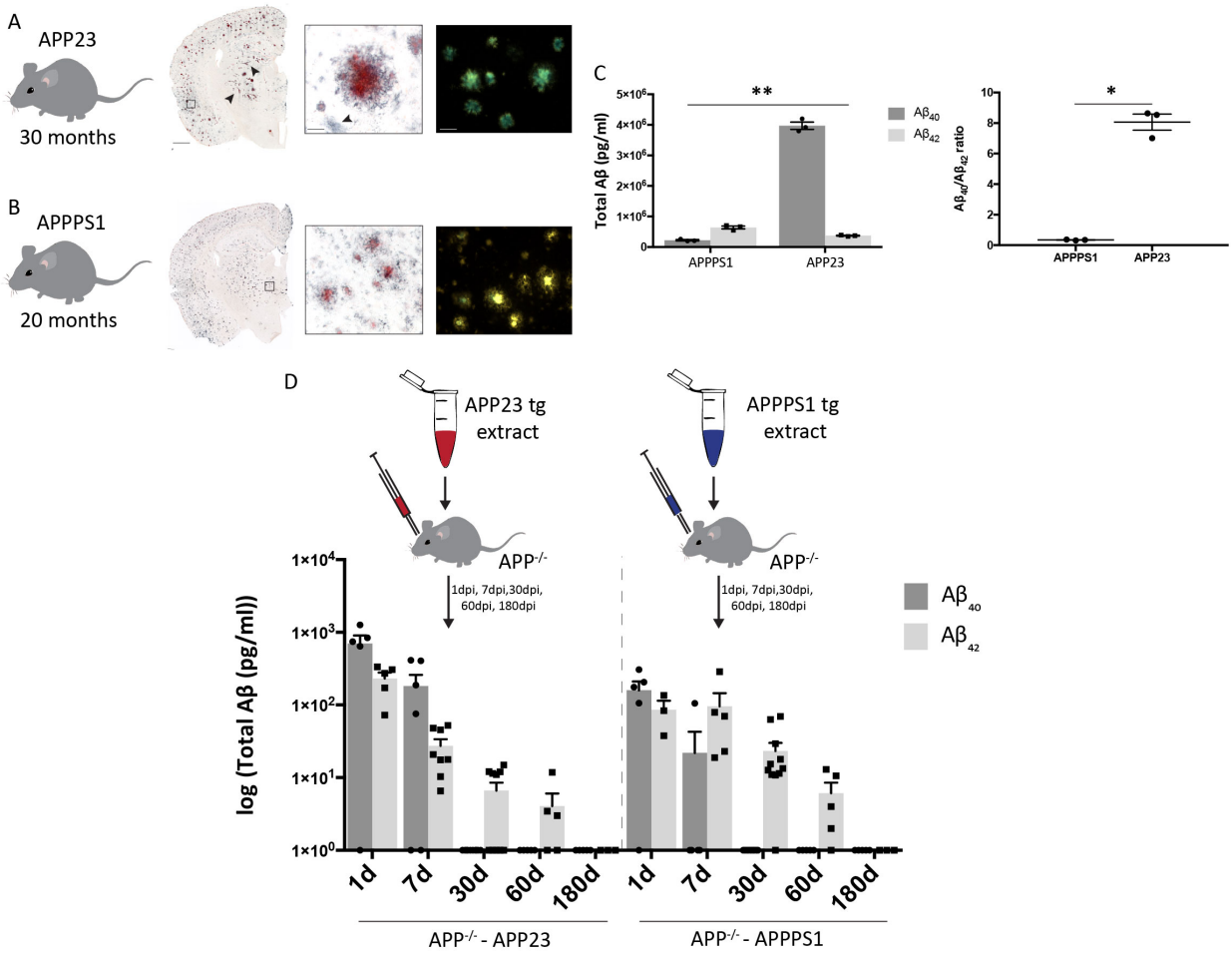

Figure 1. *Persistence towards in vivo degradation of different Aβ isoforms in APP-knockout (App$^{-/-}$) mice*. APP23 and APPPS1 mice differ in Aβ plaque morphology, spectral properties and $Aβ_{40}/Aβ_{42}$ ratios. (**A,B**) Aβ-immunostaining and Congo Red staining of a 30 months-old APP23 mouse and a 20 months-old APPPS1 mouse. APP23 mice display large congophilic plaques, diffuse plaques (arrowhead) as well as CAA (arrowheads). APPPS1 mice present with smaller, compact congophilic plaques in the absence of CAA. Staining with the LCO dyes hFTAA and qFTAA leads to greenish colour in APP23 plaques and a yellowish colour shift in APPPS1 mice. Scale bars: left 200 µm, right 50 µm. (**C**) ELISA measurements of $Aβ_{40}$ and $Aβ_{42}$ and calculation of the $Aβ_{40}/Aβ_{42}$ ratio reveal the dominance of $Aβ_{40}$ over $Aβ_{42}$ in APP23 mice and of $Aβ_{42}$ over $Aβ_{40}$ in APPPS1 mice. Indicated is the mean ± s.e.m., n=3 mice per group, two-way ANOVA for total Aβ concentration ($F(1,24)=12,65$; ** $p<0.01$); t-test for $Aβ_{40}/Aβ_{42}$ ratio ($t=14,26$; ** $p<0.01$). (**D**) A pool of Aβ-seed containing APP23 (n=3) or APPPS1 (n=3) extract was injected bilaterally into the hippocampus of 3-months old female *App*$^{-/-}$ mice. Hippocampi were dissected and biochemically analyzed 1,7, 30, 60 and 180 dpi using ultra-sensitive bead-based single-molecule array Simoa technology. Human $Aβ_{40}$ was below detection limit at 30 dpi, however human $Aβ_{42}$ could be detected up to 60 dpi and partially after 180 dpi. Indicated is the mean ± s.e.m., n=5-8 mice per group.

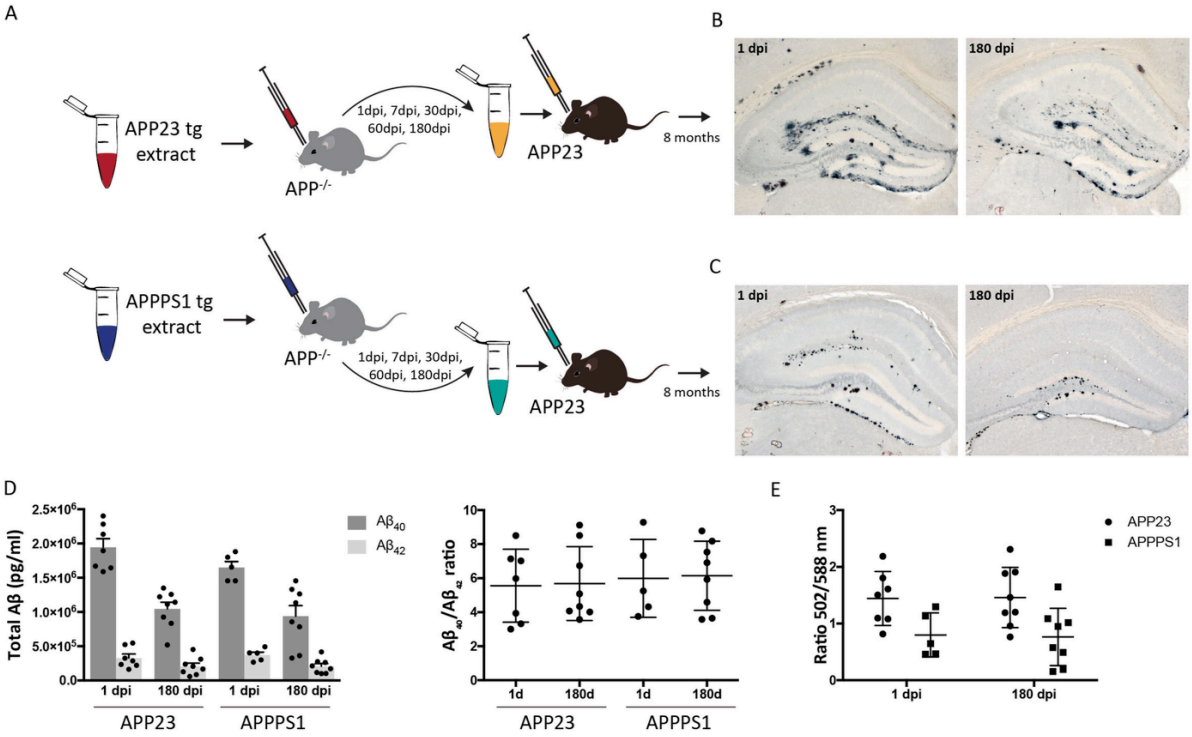

Figure 2. *Persistence of Aβ fibril polymorphism in App*$^{-/-}$ *mice for up to 6 months*. (**A**) Aβ-seed containing APP23 and APPPS1-containing brain extract was injected bilaterally into the hippocampus of 3-month-old female *App*$^{-/-}$ mice. Injected hippocampi were isolated 1, 7, 30, 60 and 180 dpi (n=5-8 mice per group), pooled and used for secondary transmission into the hippocampus of young, 2-months-old APP23 mice. (**B,C**) Brains were analyzed for Aβ deposition 8 months later. Shown are representative Aβ-immunostainings and Congo Red-stainings in mice injected with 1-dpi *App*$^{-/-}$ hippocampal extracts and 30-dpi *App*$^{-/-}$ hippocampal extracts. Interestingly, the morphological differences observed in the seeding extracts were maintained upon secondary transmission. Scale bar: 200 µm. (**D**) ELISA measurements of $A\beta_{40}$ and $A\beta_{42}$ and calculation of the $A\beta_{40}/A\beta_{42}$ ratio reveal that the host hippocampi do not reflect the $A\beta_{40}/A\beta_{42}$ ratios of the injected seeding extracts. t-test between groups revealed no significance (ns = non-significnat) (**E**) Seeded hippocampi were stained with the LCOs hFTAA and qFTAA and the ratio of the emission spectra at 502 nm and 588 nm was calculated. Mean and s.e.m. are indicated. n=5-8 mice per group, t-test revealed significant difference between the groups (t=2.199; * p<0.05).

3.4 Induction of murine cerebral β-amyloidosis as a tool to study prion-like propagation within the brain

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Induction of murine cerebral β-amyloidosis as a tool to study prion-like propagation within the brain

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Abstract

The amino acid sequence of Aβ is highly conserved between vertebrates and, consistently, age-related deposits have been described in brain of higher and long-lived mammals. An exception are mice and rats that do not develop amyloid deposits during their normal lifespan. Three amino acid substitutions at position 5 (Glutamine), 10 (Phenylalanine) and 13 (Arginine) of Aβ distinguish the mouse and human protein and this difference might contribute to absence of cerebral β-amyloidosis in mice and rats. To generate mouse models of Aβ deposition, overexpression of human APP with the Swedish double mutation has often been used, e.g. APP23 mice (Sturchler-Pierrat et al. 1997). To examine the amyloidogenic potential of mouse Aβ, we now generated a mouse model that expresses murine Aβ by mutating a previously used human APP cDNA at positions 5, 10 and 13 back to the murine amino acids at this position (APP_{swe}GFR). Mice expressing APP_{swe}GFR did not develop Aβ deposition up to 30 months of age. However, robust amyloid induction was observed 12 months after the injection of minute amounts of Aβ laden brain extract from aged APP23 mice. The induced amyloid-β deposition progressed spatially within the limbic connectome supporting the hypothesis that neuronal connections act as pathways for the spatial and temporal progression of Aβ deposition. In order to study murine vs human Aβ seeding potency and strain phenomena, we now have also crossed APP_{swe}GFR with the PS45 (PSEN1 G384A, (Herzig et al. 2004)) mouse model (APP_{swe}GFRxPS45) and these mice develop murine cerebral β-amyloidosis starting at 9 months of age. In contrast, we bred APP23 mice on an *App-knockout* ($APP^{-/-}$) background to generate a model of pure human cerebral β-amyloidosis. These tools will now allow us to study cross seeding and to follow transport and propagation of Aβ assemblies *in vivo*.

Introduction

The molecular pathogenesis of a broad range of neurodegenerative diseases involves the misfolding and subsequent aggregation of specific proteins. Evidence indicates that in the case of Alzheimer's disease (AD), the most common dementia worldwide, aggregation of the β-amyloid (Aβ) peptide triggers the fatal cascade of neurodegeneration(Hardy 2009; Hardy & Selkoe 2002; John A. Hardy & Gerald A. Higgins 1992). Compelling experimental evidence now implicates that the pathological mechanisms of the self-propagating infectious prion protein also apply to the deposition of other amyloidogenic proteins in the pathogenesis of neurodegenerative diseases (Jucker & Walker 2013). In prion diseases, physiologically soluble prion protein molecules misfold, self-assemble and spread within the nervous system by imposing their aberrant structure on native prion proteins that are produced by cellular metabolism (Aguzzi 2009; Dearmond & Prusinertt 1995; Prusiner 2013).

Cross-sectional studies on postmortem tissue of AD patients consistently indicate that the extracellular Aβ deposits develop in specific spatiotemporal patterns (Braak & Braak 1991; Brettschneider et al. 2015; Thal et al. 2006). More recently, in vivo imaging studies could show that the Aβ protein seems to spread among neuronally connected areas implicating a prion-like mechanism of spreading (Iturria-Medina & Evans 2015; Palmqvist et al. 2017). One major disadvantage of postmortem histopathological analysis is the general reliance on proteinaceous deposits that cannot provide information on the mechanisms by which the proteins spread within the brain. Intracerebral injections of Aβ-containing brain extract can induce Aβ deposition and spreading of the deposition to axonally connected areas over time (Ye et al. 2015). However, current transgenic animal models lack the ability to discriminate between the initially injected exogenous seeds and the following aggregation process.

In the present study, we made use of the fact that endogenous murine Aβ differs from its human homologue in three amino acids and developed new mouse models that either express solely murine Aβ or solely human Aβ (Kumar et al. 2013). Murine and human Aβ can therefore be distinguished by commercially available antibodies which makes it possible to observe the seeding and aggregation of both proteins in an in vivo environment. Therefore, our results provide new tools to study the prion-like propagation of the Aβ peptide in vivo and shed light on the spatiotemporal distribution in AD patients.

Methods

Generation of transgenic mouse lines

In tyrosine to phenylalanine and histidine to arginine. To generate a mouse model overexpressing murine Aβ, a previously used human APP23 cDNA was mutated to the murine Aβ sequence and introduced via

micro-injection into fertilized embryos from mice with C57BL/6 background. The mouse lines express human APP harboring the Swedish double mutation (K670N/M671L) with a murinized Aβ sequence under the neuronal Thy-1 promotor and was termed APP_{swe}-GFR. Another mouse line was generated by cross-breeding of the APP_{swe}-GFR mouse line with the PS1 G384A mouse lines, termed APP_{swe}-GFR x PS1 G384A. The mouse strain therefore expresses human APP harboring the Swedish mutation containing murinized Aβ and additionally co-expresses mutated human PS 1 (PS1 G384A), both under the neuron-specific Thy-1 promotor. All mice were group-housed under pathogen-free conditions. All experimental procedures performed were undertaken in accordance with the veterinary office regulations of Baden-Württemberg (Germany) and approved by the local Animal Care and Use Committees. this study, three new APP transgenic mouse lines were created and subsequently characterized: *APP_{swe}-GFR*, *APP_{swe}-GFR x PS1 G384A* and *APP23 x APP-knockout*. The human and murine Aβ sequence varies in three amino acids at positions 5, 10 and 13 leading to a change of arginine to glycine,

Preparation of brain tissue

Brain extracts for intracerebral injections were derived from aged depositing female APP_{swe}-GFR x PS1 G384A transgenic mice (20-25 months old) and aged depositing female APP23 x APP-knockout transgenic mice (15 – 20 months). Brain extracts for quantification of Aβ by immunoassays were prepared from male and female APP_{swe}-GFR and APP_{swe}-GFR x PS1 G384A transgenic mice aged between 1 and 25 months. Mice were euthanized with an overdose of ketamine (300mg/kg body weight) and xylazine (30mg/kg body weight) in saline. Trans-cardial perfusion was performed with ice-cold PBS for five minutes. brains were dissected and hemispheres separated by a mid-line sagittal cut. The right hemisphere was fixed in 4% paraformaldehyde in 0.1 M PBS for two days and then transferred to 30% sucrose for cryoprotection. After two days, the right hemisphere was frozen in 2-methyl-butane and stored at -80 °C for immunohistochemistry. For biochemical analyses, cerebellum and lower brainstem of the left hemisphere were removed and the forebrain was immediately fresh-frozen on dry ice and stored at -80°C until use. Tissue was then homogenized at 10% (w/v) in sterile phosphate-buffered saline (PBS, Lonza, Switzerland) using the Precelly system with a 2 × 20-s cycle at 5,500 rpm (Precellys24 Homogenizer, EQ03119- 200-RD000.0, Bertin Instruments). Afterwards, samples were vortexed and centrifuged at 3,000 x g for 5 minutes as previously described (Kumar et al. 2013). The supernatant was aliquoted and immediately frozen. For all experiments, the 10% (w/v) extract was used.

Stereotactic injection of brain extracts

Young 3-months old APP_{swe}-GFR and APP23 x APP-knockout mice were anaesthetized with a mixture of ketamine (100mg/kg body weight) and xylazine (10mg/kg body weight) in saline. Bilateral stereotactic injections of 2.5µl brain extract were performed with a Hamilton syringe that was placed into the hippocampus (AP - 2.5mm, L +/- 2.0mm. DV -1.8mm). Injection speed correlated to 1.0µl/minute and the needle was kept in place for additional 2 minutes before slowly withdrawn from

the brain parenchyma. The surgical area was cleaned with sterile saline, the incision was sutured and the mice were monitored until recovery from anesthesia and within the following weeks.

Histology and immunohistochemistry

Before staining, fixed and frozen right hemispheres were serially cut into 25 µm-thick coronal sections with the use of a freezing-sliding microtome. Collected tissue was placed in cryoprotectant (35% ethylene glycol, 25% glycerol in PBS) and stored at -20 °C until use. For immunostaining of human Aβ, the polyclonal antibody CN6 or the monoclonal antibody 7H3D6 (Kumar et al. 2013) was used. For immunostaining of murine Aβ, the polyclonal antibody Poly18058 (BioLegend Inc., California, USA) was used. Immunohistological stainings were performed according to previously published protocols (Stalder et al. 2005). Sections were counterstained with Congo Red according to standard protocols.

Histological staining with hFTAA/qFTAA and spectral analysis

For spectral analysis, coronal sections of aged APP_{swe}-GFR x PS1 G384A transgenic mice, aged APP23 x APP-knockout mice and inoculated APP_{swe}-GFR mice were stained with a combination of the LCO dyes hFTAA and qFTAA. 25 µm-thick coronal sections were washed in PBS (3x 10 min) and mounted on Superfrost slides. Sections were subsequently dried for 2 hours at room temperature. Staining with both LCOs was performed as previously described (2.4 µM qFTAA and 0.77 µM hFTAA in PBS). Sections were incubated for 30 min at RT in the dark. Spectra were acquired on a Zeiss LSM 510 META confocal microscope equipped with an argon 458-nm laser for excitation and a spectral detector (Carl Zeiss MicroImaging GmbH). A 40× oil-immersion objective (1.3 N.A.; Zeiss) was used for spectral imaging of Aβ-amyloid cores. Continuous emission spectra were acquired from 470 to 695 nm. The amyloid plaques were randomly chosen, and three regions of interest were mea- sured in the core of each deposit. Analysis was restricted to the cores of plaques. At least 20 Aβ plaque cores were measured in each mouse. For analysis, all emission spectra were normalized to their maxima and the mean spectral signature of each plaque core was calculated before averaging the values for each mouse. The ratio of the intensity of emitted light at the blue-shifted peak (502 nm) and red-shifted peak (588 nm) was used as a parameter for spectral distinction of different Aβ deposits. The peaks of the spectra were selected to maximize the spectral distinction.

Quantification of Aβ by immunoassays

For quantification of Aβ concentration, extracts were pretreated with formic acid (Sigma-Aldrich, final concentration: 70% vol/vol), sonicated for 30s on ice and centrifuged at 25,000g for 1 hour at 4°C. Supernatants were neutralized in neutralization buffer (1M Tris base, 0.5M Na_2HPO_4, 0,05% NaN_3 (wt/vol)). Human and murine Aβ was measured by an electrochemiluminescence (ECL)-linked immunoassay (Meso Sclae Discovery, MSD). Regarding the measurement of human Aβ on the MSD platform, a commercial Human (6E10) Abeta Triplex Assay was used and for the measurement of

murine Aβ, a commercial Human/Murine (4G8) Abeta Triplex Assay was used according to the manufacturer's instructions. In each case, samples and calibrators were run in duplicates. A 96-well plate pre-incubated with C-terminal capture antibodies against Aβx-38, Aβx-40 and Aβx-42 were blocked for 1 h with 1% bovine serum albumin (BSA in Tris buffer, wt/vol) and washed three times with Tris buffer. Formic acid–treated samples were co-incubated with the SULFO-TAG 6E10 detection antibody solution on the plate for 2 h. After washing, MSD Read Buffer T was added and the plate was measured immediately on the Sector Imager 6000. Data analysis used MSD DISCOVERY WORKBENCH software 2.0. Internal QC samples were used for quality control of the assay performance and inter-plate variability. Total Aβ was the sum of Aβx-38, Aβx-40 and Aβx-42.

Statistical analysis

Data in figures represent mean +/- s.e.m. Statistical analysis was performed using the GraphPad Prism software, version 6.0.

Results

The murine Aβ peptide deposits in transgenic mouse models and shows conformational differences to aggregates consisting of human Aβ peptide

The murine Aβ peptide differs from its human homologue by the substitution of three amino acids at position 5, 10 and 13. Non-transgenic rodents do not spontaneously develop amyloid deposits during their normal lifespan and the intracerebral infusion of Aβ seeds does not induce fibril formation over prolonged incubation periods. Human and murine Aβ peptides differ by the substitution of three amino acids at position 5, 10 and 13 which might cause the observed absence of cerebral β-amyloidosis in rodents (Figure 1).

To evaluate the amyloidogenic potential of the human and murine Aβ peptide, we generated three new mouse models: APP_{swe}-GFR, APP_{swe}-GFR x PS1 G384A and APP23 x APP-knockout. Both the APP_{swe}-GFR and APP_{swe}-GFR x PS1 G384A mouse line express exclusively murine Aβ. The APP_{swe}-GFR x PS1 G384A strain additionally expresses the PS1 mutation G384A. Although both mouse strains express murine Aβ with a three-fold overexpression, only the APP_{swe}-GFR x PS1 G384A mouse line exhibits deposition at around 10 months of age. APP_{swe}-GFR mice demonstrate a high $Aβ_{40}/Aβ_{42}$ ratio. On the contrary, the addition of the PS1 G384A mutation causes a shift of the $Aβ_{40}/Aβ_{42}$ ratio that likely represents the trigger for the deposition of murine Aβ. (Figure 1). In comparison to the purely human plaques in the APP23 x APP-knockout mouse line that appear compact and Congo Red positive, amyloid deposits made up of murine Aβ have a rather diffuse morphology and are rarely Congo Red positive. LCO analysis using the fluorescent dyes hFTAA and qFTAA support the morphological differences observed by histology and may indicate conformational polymorphism (Figure 2).

Introduction of exogenous seeds causes plaque deposition in the APP_{swe}-GFR host in a prion-like mechanism

Human APP23 x APP-knockout brain homogenate as well as murine APP_{swe}-GFR x PS1 G384A brain extract was bilaterally injected into the hippocampus of an APP_{swe}-GFR host. Twelve months post injection, both extracts induced robust seeding and strain-like morphologies of the injected material could be partially conveyed by seeded transmission. Structural polymorphisms manifested in histological plaque appearance and differences in binding to the fluorescent amyloid dyes hFTAA and qFTAA. Interestingly, while the APP23 extract-induced Aβ deposits displayed a typical emission spectrum, the APP_{swe}-GFR x PS1 G384A extract-induced Aβ deposits showed a very weak binding to the amyloid dyes (Figure 2). In both cases, the depositing plaque material was entirely made of murine Aβ, and therefore originated from the host environment. These results were confirmed by histological stainings as well as ECL-linked immunoassays (Figure 2).

Furthermore, the injected brain homogenate does not only cause Aβ depositions in the focally injected area, the hippocampus, but propagates to axonally connected areas over time. In case of the APP_{swe}-GFR mouse strain, these brain areas include the entorhinal cortex, the frontal cortex and olfactory bulb, the fornix, the mammillary bodies, extensive CAA in the meninges as well as parts of the parietal and temporal cortex above the injection site. Regarding the temporal pattern of plaque deposition, strong deposition of CAA in the meninges appears six months post injection, followed by hippocampal plaque deposition around 8 months post injection. After 10 months, the entorhinal cortex becomes increasingly involved and at around 12 months post injection, Aβ deposition becomes visible in the anterior olfactory bulb and frontal cortex (Figure 3). These results point towards an implication of axonally connected areas in the spatiotemporal propagation of Aβ deposits within the brain.

Discussion

Most age-related neurodegenerative diseases are human-specific and rarely occur spontaneously in other animals. To study these diseases and to gain insights into the onset of pathologies, genetically manipulated mouse models are used. Most of the currently used transgenic mouse models overexpress mutant human APP, mutant PS1 or a combination of both (Jucker 2010; Walker & Jucker 2017). Additionally, these transgenic mice generate endogenous murine Aβ that has been shown to co-aggregate with human Aβ into amyloid deposits (Mahler et al. 2015).

Here we describe the development and characterization of two new mouse models that exclusively express murine Aβ. The APP_{swe}-GFR mouse model expresses a high $Aβ_{40}/Aβ_{42}$ ratio and shows no plaque deposition its lifetime, the additional expression of the PS1 G384A mutation within the APP_{swe}-GFR x PS1 G384A mouse model causes a lower $Aβ_{40}/Aβ_{42}$ ratio and plaque deposition at around 10 months of age. The higher concentration of the $Aβ_{42}$ isoform is likely the trigger for the deposition of

murine Aβ. These results point towards the importance of the $A\beta_{42}$ isoform as the initial seed in the context of amyloidogenic deposition. Therefore, rodent-sequence Aβ is capable of aggregation in the living brain. One possible explanation why wild-type rodents lack senile plaques as they age is a different processing of the APP protein. Wild-type mouse APP is preferentially cleaved by BACE1 at the +11 amino acid site generating non-amyloidogenic $A\beta_{11\text{-}40/42}$ (Cai et al. 2001; Chow et al. 2010). In our mouse models, the combination of familial mutations shifting the BACE cleavage site to +1 and an increase in the $A\beta_{42}$ concentration likely causes the observed plaque induction. The comparison between entirely human and solely murine Aβ deposits revealed striking differences in the respective plaque morphologies. While murine Aβ plaques are of a diffuse nature and rarely Congo Red positive, human Aβ plaques presented as large, compact and Congo Red positive. Subsequent analysis using the fluorescent dyes hFTAA and qFTAA supported the morphological differences observed by histology. Indeed, the morphological differences could be propagated upon seeded transmission and indicate conformational polymorphism.

We further studied if Aβ aggregation in the non-depositing APP_{swe}-GFR mouse line can be induced by intracerebral injection of either human or murine seeds. After an incubation time of twelve months, robust plaque induction was visible for both extracts that were injected. This confirms recent in vitro and in vivo results that human and murine Aβ is able to cross-seed and form mixed fibrils (Fung et al. 2004; Morales-Corraliza et al. 2013; Pype et al. 2003; van Groen et al. 2006). Importantly, in both cases, the depositing plaque material was entirely made of murine Aβ, and therefore originated from the host environment. The results manifest the implication of the prion paradigm for the seeded aggregation of the Aβ protein in vivo: In the presence of an adequate seed, either human or murine, the templated misfolding of soluble endogenous protein, in this case murine Aβ, is induced and deposition becomes histologically observable after a lag phase of a variable time span (Jucker & Walker 2013, 2018).

Furthermore, the injected brain homogenate did not only cause Aβ depositions in the focally injected area, the hippocampus, but propagated to axonally connected areas over time resembling the spread of prion lesions within the nervous system. Similar to earlier studies, the predominant neuroanatomic pattern of emerging Aβ deposition seems to follow the neuronal connectivity of the limbic system (Ye et al. 2015a).

Neurodegenerative diseases have a long, quiet phase during which the abnormal proteins proliferate in the nervous system, long before characteristic lesions can be detected with biochemical of histological tools and long before first clinical symptoms of the disease present (Bateman et al. 2012; Jack & Holtzman 2013; Ye et al. 2017). However, in terms of therapeutic prevention, this phase offers the best time frame for treatment options and therefore it is important to understand the mechanisms of protein spreading within the brain. It is currently not known if the spatiotemporal spreading of protein deposits

in AD represents an axonal spreading to specific brain regions or rather a selective vulnerability of specific brain areas. In the future, the mouse models described in this study will be used to follow the transport and propagation of human Aβ assemblies (APP23 x APP-knockout) from the injection site to the surrounding brain parenchyma of the murine mouse model (APP_{swe}-GFR).

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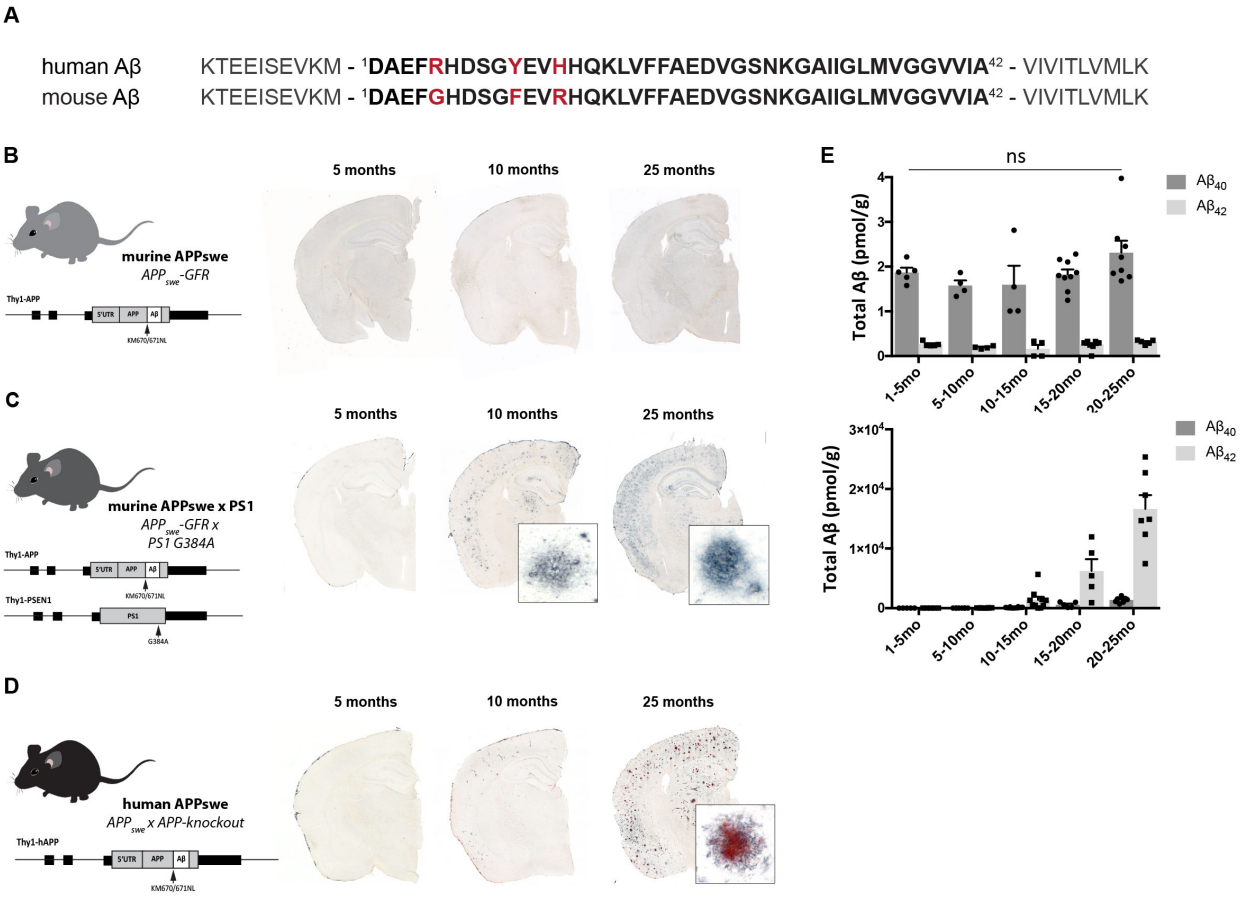

Figure 1. *The mouse lines APP$_{swe}$-GFR and APP$_{swe}$-GFRxPS45 show different Aβ aggregation potentials*. (**A**) Comparison of human and mouse Aβ sequences. The 3 sequence differences between human and mouse Aβ at residues 5, 10 and 13 are depicted in red. (**B,C**) Two different transgenic mouse lines overexpressing mouse Aβ were generated. APP$_{swe}$-GFR expresses human APP harboring the Swedish double mutation (K670N/M671L) with a murinized Aβ sequence under the neuron-specific Thy-1 promotor. APP$_{swe}$-GFRxPS45 additionally co-expresses mutated human PS 1 (PS 1 G384A) under the neuron-specific Thy-1 promotor. Brain sections were stained with the Aβ-specific CN6 antibody and Congo Red. Representative images of the midbrain region at either 5, 10 or 25 months of age are shown. The APP$_{swe}$-GFR mouse line shows no plaque deposition at the age of 25 months, the APP$_{swe}$-GFRxPS45 mouse line shows first plaques at the age of 8 months. Plaques appear to have a diffuse nature and are not Congo Red positive. (**D**) APP23 mice were crossed with APP-knockout mice. The *APP23-APP-knockout* mouse line expresses entirely human Aβ. First plaques deposit around 8 months of age. Plaque morphology appears large, compact and Congo Red positive. (**E**) APP$_{swe}$-GFR and APP$_{swe}$-GFRxPS45 FA- extracted Aβ$_{40}$ and Aβ$_{42}$ levels were measured by an electrochemiluminescent-linked immunoassay. Results revealed a higher Aβ$_{40}$/Aβ$_{42}$ ratio in the APP$_{swe}$-GFR mouse line that does not increase over time and a lower Aβ$_{40}$/Aβ$_{42}$ ratio in the APP$_{swe}$-GFR x PS45 mouse line that increases with age. Indicated is the mean ± s.e.m., n=4-8 mice per group. t-test between groups revealed no significance (ns = non-significnat)

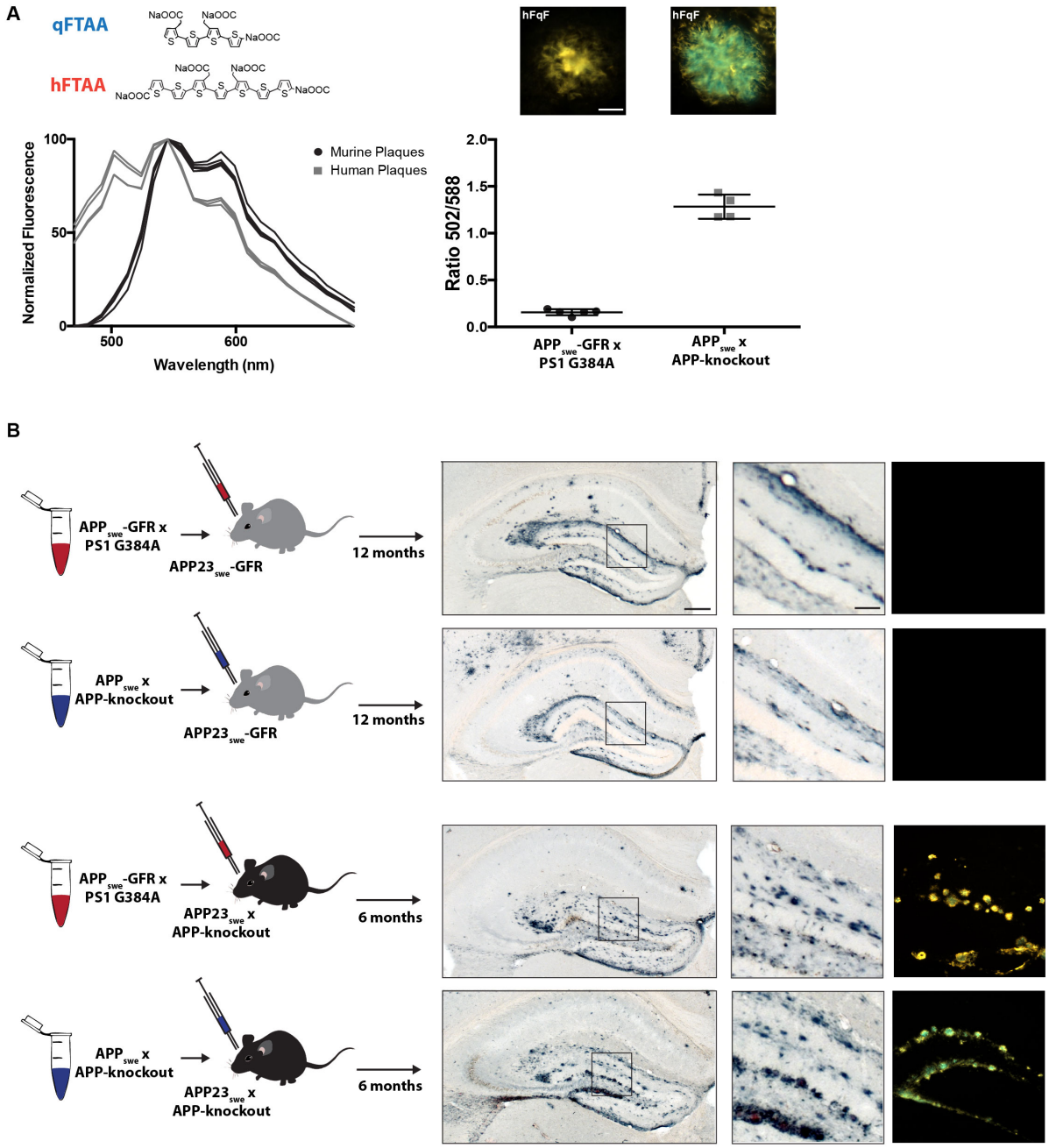

Figure 2. *Human and murine Aβ plaques present fibril polymorphism*. (**A**) Combination of two LCOs, qFTAA and hFTAA, was used to stain Aβ plaques in APP_{swe}-GFRxPS45 and APP23 null mice. Plaques were randomly selected, and for each plaque core,the fluorescence intensity was measured at 22 wave-lengths. The ratio of fluorescence intensity at 502 nm and 588 nm was calculated. Indicated is the mean ± s.e.m., n=5 mice per group, t-test revealed significant difference between the groups (t=19.13; *** p<0.0001). (**B**) Brain extracts from aged $APP23_{swe}$-GFR x PS1 mice and APP23 null mice were injected into the hippocampus of $APP23_{swe}$-GFR mice. $APP23_{swe}$-GFR mice were 2-3 months old when they were injected. A robust amyloid induction could be induced 12 months after the injection. Mice were stained with the Aβ-specific CN6 antibody and Congo Red. The APP23 null extract-induced deposits are more punctate and compact compared with the diffuse deposits induced by the murine extracts.

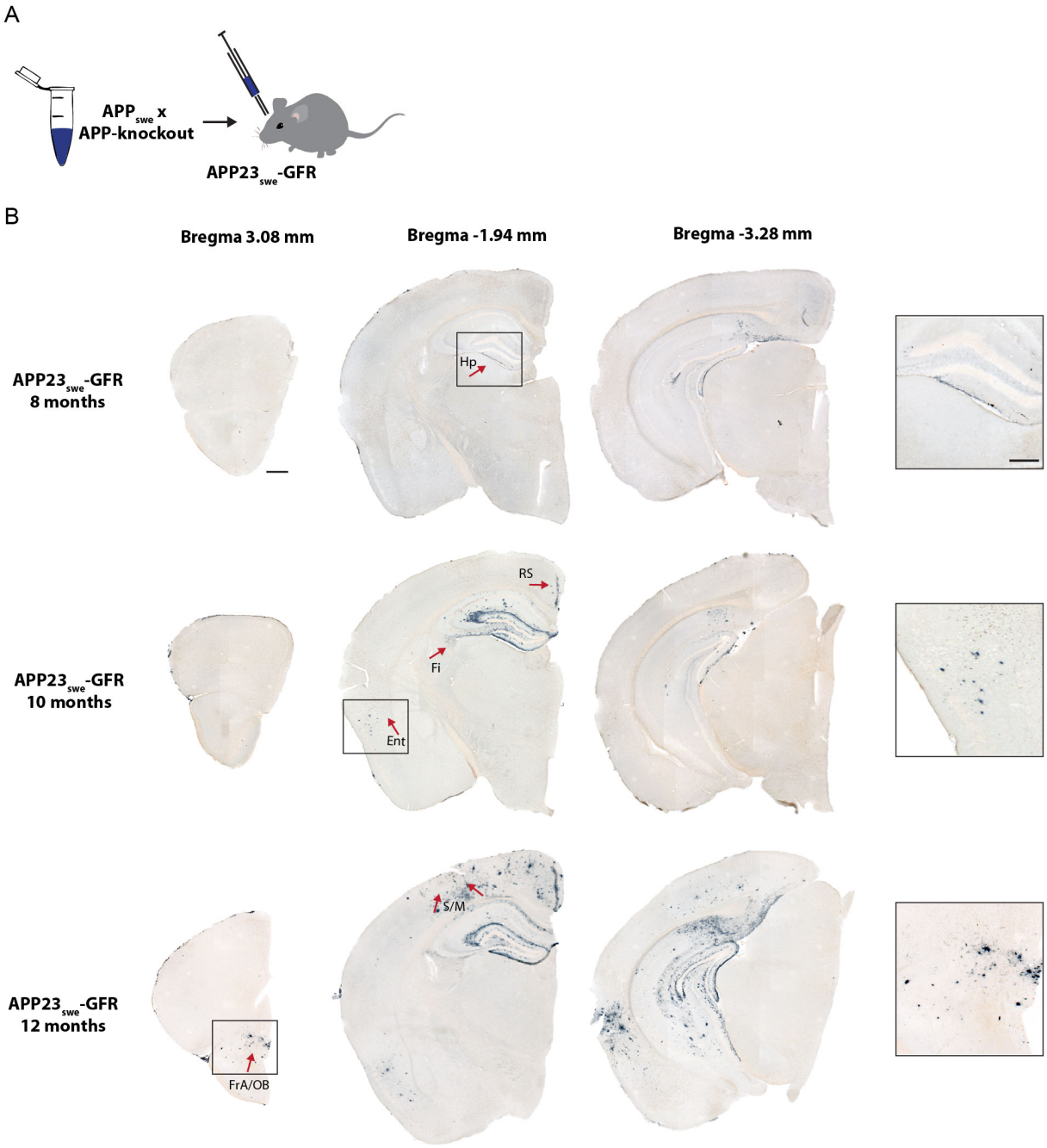

Figure 3. Brain extracts from aged APP23$_{swe}$-GFR x PS1 mice were injected into the hippocampus of APP23$_{swe}$-GFR mice. APP23$_{swe}$-GFR mice were 2-3 months old when they were injected. Immunohistochemical staining shows the regional distribution of the Aβ deposits after 8 months, 10 months and 12 months in different areas of the brain. (Hp, Hippocampus; Ent, Entorhinal Cortex; RS, Retrosplenial area; Fi, Fimbria; FrA, Frontal area ;OB, Olfactory Bulb; S, somatosensory cortex; M, motor cortex).

4. Appendix

4.1. Abbreviations

Aβ, $Aβ_{40}$, $Aβ_{42}$	β-amyloid, 40 amino acid variant, 42 amino acid variant
aa	amino acid
AD, fAD, sAD	Alzheimer's disease, familial AD, sporadic AD
AChE	Acetylcholinesterase
ADAM10	a disintegrin and metalloproteinase domain containing protein 10
AICD	APP intracellular domain
ALS	Amytrophic lateral sclerosis
APLP	Amyloid precursor like proteins
Aph-1	anterior pharynx-defective *1*
ApoE	Apolipoprotein E
APP	Amyloid Precursor Protein
BACE1/2	β-site APP cleaving enzyme 1/2
BSE	bovine spongiform encephalopathy
CAA	cerebral amyloid angiopathy
C83, C99	C-terminal fragment 83, C-terminal fragment 99
CJD	Creutzfeldt-Jacob disease
CSF	cerebrospinal fluid
ECL	electrochemiluminescence-linked
EOAD	early onset AD
EPR	electron paramagnetic resonance
GPI	glycosylphosphatidylinositol
GSS	Gerstmann-Sträussler-Scheinker syndrome
GSK3β	glycogen synthase kinase 3β
GWAS	genome wide association study
HD	Huntington's disease
kDa	kilodalton
LCO	luminescent conjugated oligothiphene
LOAD	late onset AD
MAPT	microtubule-associated protein tau
Nct	nicastrin
NTF	N-terminal fragment
PCA	posterior cortical atrophy
PD	Parkinson's disease

Pen-2	PSEN enhancer 2
PET	positron emission tomography
PiB	Pittsburgh compound B
PS	Presenilin
PrP^{C}, PrP^{Sc}	cellular prion prion protein, misfolded prion protein
ssNMR	solid-state nuclear magnetic resonance
Thy-1	thymocyte differentiation antigen 1
TM	transmembrane
TSE	transmissible spongiform encephalopathy