Crossing barriers, delivery of llama antibody fragments into the brain

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CHAPTER

GENERAL INTRODUCTION

ABSTRACT

Llama antibody fragments (VHH) have the intrinsic potential to be developed as diagnostic, therapeutic and even theragnostic tools in a wide variety of diseases and afflictions. Their potential application includes the diagnosis and treatment of neurodegenerative disorders, among which Alzheimer's disease (AD). However, currently this application is hampered by, amongst other factors, the blood-brain barrier (BBB). The BBB strictly regulates what goes into and out of the brain. Drugs that can theoretically be used to detect or fight neurodegenerative disorders often do not cross this barrier. Yet delivery of compounds into the brain is of vital importance for both detection and treatment of cerebral diseases such as AD. This thesis focusses on the delivery of VHH into the brain. It discusses a variety of delivery methods and provides a platform for both diagnostic and therapeutic applications of VHH in neurodegenerative disorders in general and in particular in dementia and AD.

1. INTRODUCTION

1.1 Dementia & Alzheimer's disease

Dementia is a syndrome caused by a number of progressive illnesses that affect memory, thinking, behavior and the ability to perform everyday activities. With age being the major risk factor, prevalence of dementia above the age of 65 doubles with every five year age increments. Currently, an estimated 44 million patients worldwide are affected by dementia, a number expected to double by 2030 and more than triple by 2050 [1].

AD is the most prevalent (50-75%) form of dementia. It is clinically characterized by a gradual decline in memory and cognitive function. Early clinical symptoms of AD, e.g. short-term memory loss, steadily progress to more extensive cognitive and emotional dysfunction. Eventually, the patient becomes completely bedridden and dependent on fulltime professional care for activities of daily living. Death, as a direct result of the disease, occurs on average nine years after the moment the first clinical symptoms can be noticed in the patient. Due to the devastating and relentless nature of the affliction, dementia in general and AD in particular are one of the main causes of dependence and disability at older ages, one for which no cure has yet been developed [2].

AD has two main pathological hallmarks, i.e. intercellular neurofibrillary tangles (NFT) of hyperphosphorylated tau protein and extracellular aggregates of the peptide amyloid beta (Aβ). Aggregates of Aβ can occur in senile or diffuse plaques in the neuropil, or in the vasculature of the cerebral cortex. Cerebrovascular deposition of Aβ in the vessel walls (*tunica media*) of the parenchymal and leptomeningeal arteries and arterioles is referred to as cerebral amyloid angiopathy (CAA), with a subset of CAA involving deposition around capillaries (capCAA). CAA occurs in approximately 70% up to 100% of AD patients [3–6]. Other neuropathological changes associated with AD include pronounced atrophy of certain brain regions, substantial enlargement of the cerebral ventricles, widening of the cortical sulci and narrowing of the gyri [7], see also Figure 1.1. The yet irreversible, and damaging, neuropathological changes start as early as two to three decades before cognitive decline is clinically noticeable [8–10], and accumulation and aggregation of Aβ is currently thought to be the first of all these pathological changes to occur [11].

1.2. Aβ and AD

Formation of the peptide Aβ is the result of one of the two posttranslational pathways of sequential cleavage of its precursor protein, the type-1 transmembrane glycoprotein APP (human amyloid beta A4 precursor protein) [12,13]. Cleavage of APP follows either the amyloidogenic or the non-amyloidogenic pathway. In the amyloidogenic pathway APP is sequentially cleaved

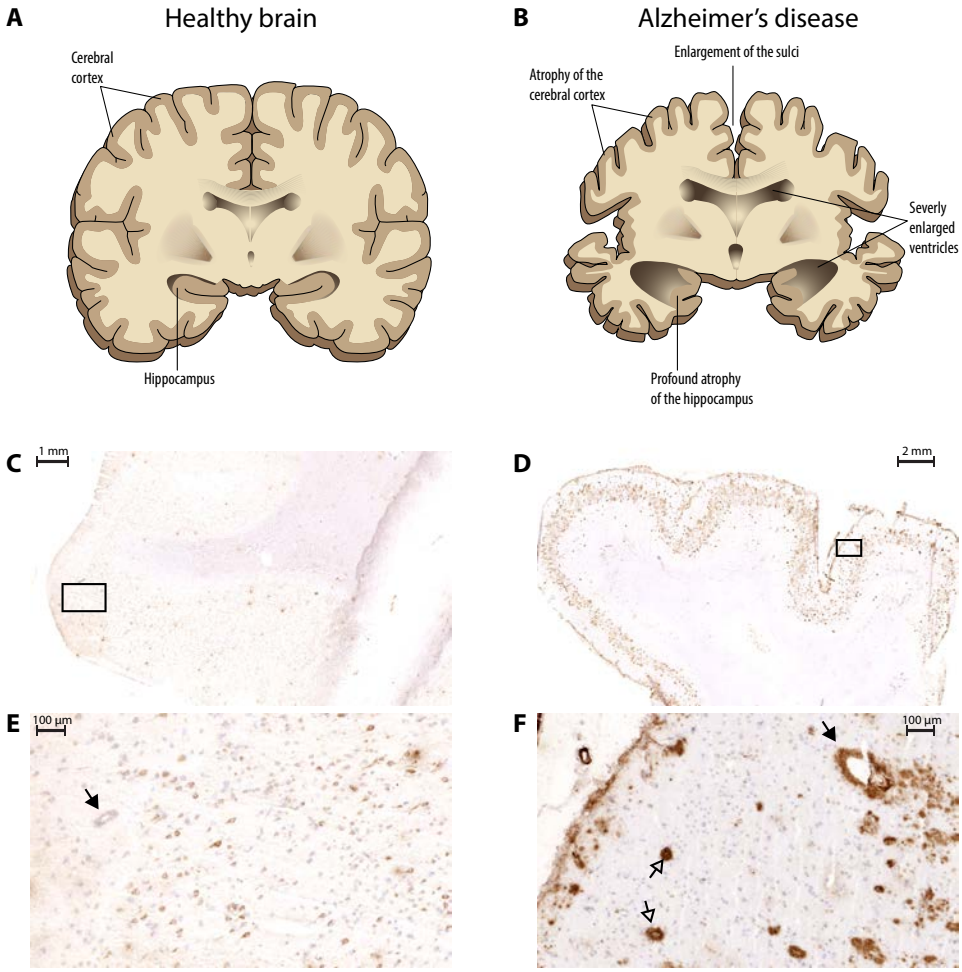

Figure 1.1. One of the most evident neuropathological changes associated with Alzheimer's disease is extreme atrophy of the brain. The sulci, or grooves, pressed together by the gyri of the healthy brain **(A)**, are enlarged in the brain of patients as the gyri atrophy **(B)**. Also the cerebral ventricles become bigger and significant shrinkage of the hippocampus, the short-term memory control center of the brain, is observed. On a microscopic scale, depositions of the aggregated peptide amyloid beta (Aβ) is visible in the parenchyma (open arrows) and the around the cerebral vasculature (closed arrows) in the diseased brain **(D and F)** but not in the healthy age matched brain **(C and E)**. Figures A and B are modelled after scientific images from the National Institute on Aging/National Institutes of Health, Bethesda, MD, USA. Parts **C-F** are 5 µm thick cryosections stained with murine monoclonal anti-Aβ antibody 4G8 **(C and E)** and the anti-Aβ VHH pa2H **(D and F)** following methods described in chapters 2-5 in this thesis.

by first the β-secretase followed by the γ-secretase and results in the formation of the peptide Aβ. On the other hand, in the non-amyloidogenic pathway APP is cleaved by the α-secretase before cleavage by the γ-secretase and is consequently cut into putatively harmless fragments [14–16], Figure 1.2. See also Box 1.1. The Processing of APP for more details.

Following the amyloidogenic pathway, the Aβ fragment can range in size from 38 to 43 amino acids. Most of the Aβ that gets produced is 40 amino acids long, Aβ40, followed by Aβ42. The exact physiological function of Aβ, of any length, in both healthy and diseased states of the

Box 1.1. The Processing of APP

The type-1 transmembrane glycoprotein APP is processed by sequential cleavages in either the non-amyloidogenic or the amyloidogenic pathway, and only the latter leads to Aβ.

In the non-amyloidogenic pathway APP is first cleaved by α-secretase. The activity of α-secretase is mediated by one or more of the 26 enzymes from the family of disintegrin and metalloproteinase domain proteins (ADAM), with most evidence pointing towards either exclusively or mainly ADAM10 [17,18]. The α-secretase cleaves APP between its Lys683 and Leu684 residues, or the 16th and 17th amino acid inside what could have become the Aβ fragment [15,19]. This divides APP into a large soluble extracellular domain (sAPP-α) and a membrane-associated C-terminal fragment of 83 amino acids (C83, or CTF-α). The CTF-α fragment is then cleaved by the γ-secretase, a large proteinase complex made up by presenilin (PS1 or PS2), nicastrin, presenilin enhancer 2 (PEN2), and anterior pharynx-defective 1 (APH1) [20–23]. The γ-secretase complex cleaves the CTF-α fragment into the P3 peptide and the C-terminal APP intracellular domain (AICD). The two smaller fragments may likely have functions in downstream signaling pathways, including a role for the AICD in a negative-feedback loop for the γ-secretase complex, but their exact functions are still poorly understood [24,25].

In the amyloidogenic pathway, which eventually leads to Aβ, APP is first cleaved by β-secretase, a single enzyme also known as BACE1 [26–29]. BACE1 can cleave APP at two different sites, i.e. either between Met596 and Asp597 of APP (the Asp-1 cleavage site) or between Tyr606 and Glu607 (the Glu-11 cleavage site), where the Glu-11 site is the preferred site *in vivo* and *in vitro* [30]. The BACE1 cleavage produces a secreted, extracellular sAPP-β and either a 99 or 89 residue membrane associated CTF-β (C99 or C89, respectively). The CTF-β fragments are subsequently cleaved by the γ-secretase complex creating the intracellular AICD and, in the case of C99, the amyloidogenic Aβ peptide.

While α-secretase and β-secretase both have only one or two very precise cleavage sites, the γ-secretase complex can cleave the membrane-associated CTF-β at a variety of locations within its transmembrane domain. Therefore, the resulting Aβ peptide can vary in length between 38 and 43 amino acids *in vivo*. Longer variants of Aβ are known, e.g. Aβ46 and Aβ49 [31]. However, they may be the product of pathways involving other secretases, aptly named ε- and ζ-secretase. The functions of the longer peptides are as of yet unknown and they are likely intermediate peptides before being further cleaved to Aβ peptides of 40 and 42 amino acids in length (Aβ40 and Aβ42, respectively) [32,33]. Peptides of these two lengths, Aβ40 and Aβ42, are the two most common forms of the Aβ fragment found *in vivo*; especially Aβ40, which accounts for approximately 90% of the produced Aβ variants [34,35].

Finally, which of the processing pathways APP follows depends on its subcellular localization as it can encounter the different secretases at different locations throughout the cell and the cell surface. Only a small proportion of APP is found on the cell surface, and over 50% is internalized within 10 minutes and sorted into early endosomes (reviewed by Zhang et al. [36]). BACE1 and APP interact predominantly in the early endosomes, while the α-secretase predominantly acts on the cell surface. Trafficking of APP is subjected to a number of different sorting and transporting proteins, among which members of the Vps10p-domain receptor family, Sortilins [37]. Removal of existing, aggregated Aβ has become a controversial therapeutic target, as promising antibody-based candidates show both negative (e.g. bapineuzumab in two phase 3 randomized trials; NCT00574132 and NCT00575055 [38]) as well positive results (e.g. aducanumab in the phase 1b randomized trial PRIME; NCT01677572 [39]) in clinical trials. As a result, general research interest should be and has been including modulation of Aβ production and aggregation via any of the targets within the APP processing pathways, be it the secretases themselves, the cleavage sites on the protein or sorting of the protein into more favorable compartments [40].

brain is still debated. Fragments of Aβ have been correlated to a variety of functions, including neurogenesis, pro-inflammatory response, long-term potentiation, and anti-microbial and anti-viral functions [41–46]. Indeed, soluble monomeric Aβ is not considered a threat for

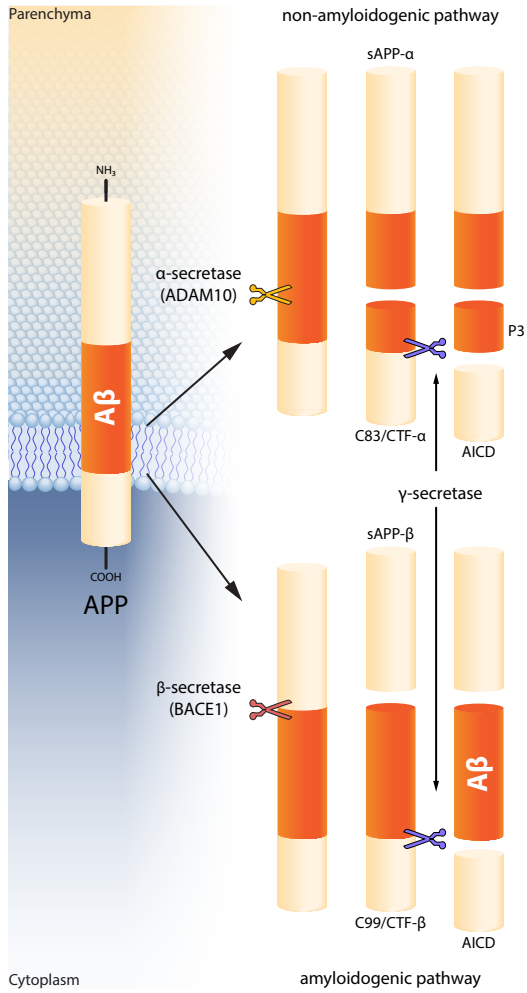

Figure 1.2. Amyloid precursor protein (APP) is a type 1 transmembrane protein with the majority of the protein exposed to the extracellular space, i.e. brain parenchyma in the case of neuronal expression. APP follows sequential cleavage in either the non-amyloidogenic pathway **(top)** or the amyloidogenic pathway **(bottom)**. APP gets cleaved by either α-secretase (yellow scissors) or β-secretase (red scissors), releasing a soluble fragment called sAPP-α and sAPP-β respectively. The membrane-bound fraction then gets cleaved by the γ-secretase complex in either pathway. In the non-amyloidogenic pathway this second cleavage creates a P3 fragment and an APP Intracellular Domain (AICD). In the amyloidogenic pathway, the second cleavage releases the amyloid beta (Aβ) peptide, which eventually will aggregate into deposits in the brain parenchyma, in the *tunica media* of the vessel walls and around capillaries.

neuroviability; the problem arises when the peptide starts to self-aggregate. The longer Aβ42 is much more prone to aggregation and is more abundant in the characteristic amyloid plaques found in AD, despite being produced significantly less than Aβ40 [34,47]. The propensity of Aβ42 to aggregate is influenced by the additional two amino acids on the C-terminus of the Aβ42 peptide (i.e. I41 and A42) which make Aβ42 both more hydrophobic and significantly more rigid than Aβ40. The increased hydrophobicity favors aggregation to reduce exposure of the hydrophobic tail and the increased rigidity makes aggregation entropically affordable [48].

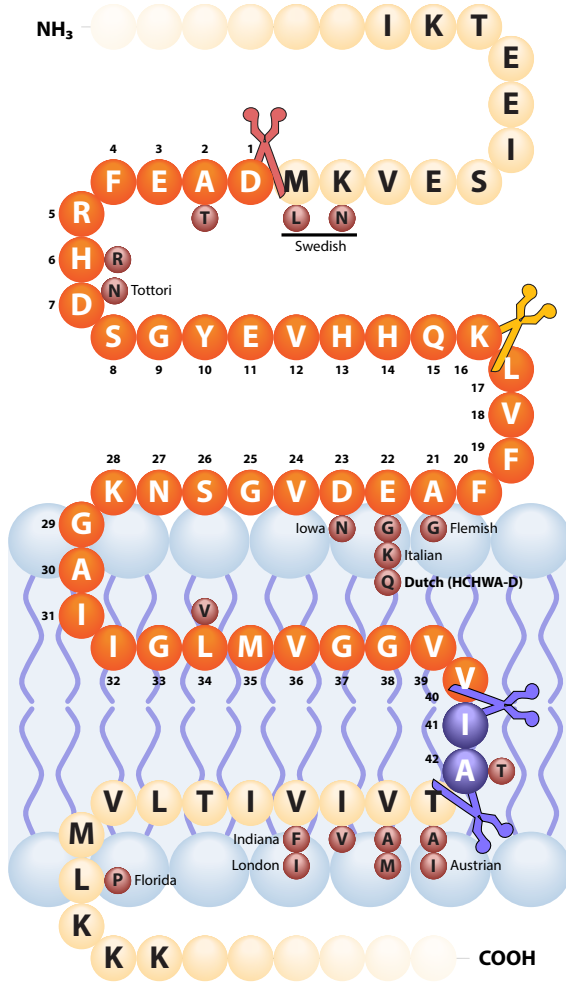

Figure 1.3. Mutations on the amyloid precursor protein (APP) in and around the 40-42 amino acid long Aβ fragment (indicated in orange) lead to altered processing of the transmembrane protein and can change the rate at which Aβ is formed, the ratio between Aβ40 and 42, and the propensity of the Aβ fragments to aggregate inside the brain. A few known point mutations are indicated by the smaller, red bubbles underneath the corresponding consensus sequence. The names next to a number of these mutations correspond to the location where the mutation was first described, or originates from, or to which location it is generally restricted. For example: E22Q corresponds to the mutation leading to HCHWA-D and both originates in, and is predominantly restricted to, a municipality in the Netherlands (i.e. Dutch). Numbering is based on the start of the Aβ fragment, immediately following the cleavage site for BACE1 (indicated by the red scissors), i.e. Aβ fragments start at D1. Calculated from the start of the most common form of APP, the Aβ fragment would start at D672. Yellow scissors indicate the cleavage site of α-secretase and blue scissors indicate two possible cleavage sites for the γ-secretase complex, leading to the production of Aβ40 and Aβ42. APP breaches the cellular membrane at the indicated locations, e.g. between K28 and G29. Not all known single nucleotide polymorphisms are indicated, and not all known nomenclature is supplied. Suggested secondary structure is not based on factual data and is solely designed to ensure proper fitment within the figure.

For almost three decades, aggregation and deposition of Aβ has been postulated as the initial causative event in the formation of Alzheimer's disease, a theory called the amyloid cascade hypothesis [49]. The many genetic variations found on the APP gene that cause altered APP

Box 1.2. APP genetics and HCHWA-D

The importance of Aβ in the development of AD is underlined by the presence of autosomal dominant genetic risk factors found on the genes for both APP and members of the γ-secretase complex [50]. A plethora of point mutations have been described for the APP gene, each having a slightly different effect on Aβ production and aggregation [51]: some increase total Aβ production by promoting cleavage by BACE1 over the α-secretase (e.g. Swedish mutation KM670/671NL [52]), some favor production of Aβ42 over Aβ40 (e.g. Leuven mutation E682K [53]), or the other way around (e.g. Italian mutation E693K [54]), some increase the propensity to form harmful aggregates and even one mutation has been found to be protective against aggregation of Aβ (i.e. A673T [55,56]), Figure 1.3. Also the other proteins involved in the formation of Aβ have been found to carry genetic risk factors. For example, over 180 point mutations have been described for the gene encoding for PS1, one of the members of the γ-secretase complex [51]. Most of these mutations, such as one leading to the deletion of exon 9 of PS1 [57], have clear pathogenic effects on AD formation. Many of these mutations have been introduced in mice in order to model for AD [58–60]. An example is the mouse model APPswe/PS1dE9 (APP/PS1) [61]. The APP/PS1 mouse develops Aβ aggregates as early as 6 months of age, and shows signs of spatial and memory deficits at 12 months [62–64]. The APP/PS1 mouse model has been used as a model for the disease throughout the studies described in this thesis.

One mutation in APP, E693Q, leads to a variant of severe CAA with secondary haemorrhages and infarcts at a relatively early age of onset (40-50 years) [65–68]. This variant is called Hereditary Cerebral Haemorrhage With Amyloidosis – Dutch-type (HCHWA-D). HCHWA-D is found in a demographic mostly restricted to the coastal village Katwijk in the Netherlands. The Aβ peptide derived from this mutated APP (counting from the Asp1 cleavage site of BACE1, the mutation becomes E22Q in the Aβ peptide) is more prone to aggregation and more resistant to degradation by proteolytic enzymes compared to the wildtype variant [69–72]. Importantly, clearance from the brain into the blood stream at the cerebral vasculature is significantly less efficient for the E22Q-bearing Aβ peptide than for the wildtype variant [73]. This reduced clearance creates a permeation barrier in the BBB endothelium, which can explain why Aβ aggregation in HCHWA-D is predominantly found in the cerebral vasculature, rather than in the parenchyma [73,74]. Over the years, the Leiden University Medical Center and others have done much research into the etiology, the diagnosis and the possible treatments of particularly this variant of CAA [75] and in recent years The Dutch CAA Foundation has been initiated to increase public exposure and raise research funding for research into CAA in general and HCHWA-D in particular.

processing and lead directly to onset of familial hereditary AD variants, strongly corroborate the amyloid cascade hypothesis (see also Box 1.2. APP genetics and HCHWA-D).

Scientific focus on the amyloid cascade hypothesis has driven initial research into AD therapeutics mainly towards anti-Aβ applications [76]. Large-scale anti-Aβ antibody-based clinical trials have been started in the first decade of the 21st century and at least one of them, the solanezumab trial, showed that the use of antibodies may indeed mobilize Aβ (especially Aβ42) from amyloid plaques [77]. More recently, the PRIME study, a phase 1b clinical trial involving the antibody aducanumab directed specifically against aggregated Aβ, showed very promising dose- and time-dependent reduction of soluble and insoluble Aβ in the brains of patients with prodromal or mild AD [39]. However, other anti-Aβ antibody trails further down the clinical trial procedures, such as phase 3 bapineuzumab trials, did not improve clinical outcomes in patients with mild to moderate AD (NCT00574132; NCT00575055) [38,78]. The varying results

of the trials led to question the hypothesis and many agree that the original amyloid cascade hypothesis as linear causality is an oversimplification [40,79,80]. Nonetheless, Aβ remains the main target of high interest for pharmacological intervention [81]. As such, solanezumab continues to be tested as a promising therapeutic approach, specifically for patients with mild AD (ExpeditionPRO, NCT02760602)[82]. Also the PRIME study continued and started recruiting participants for the phase 3 clinical trial of aducanumab in 2016 (ENGAGE, NCT02477800 and EMERGE, NCT02484547). Furthermore, anti-Aβ therapeutics may be of pivotal importance in the fight against the familial hereditary AD variants, in which aberrant processing of APP as a result of mutations invariably leads to increased aggregation of Aβ accompanied by early onset dementia (i.e. onset < 65 years of age). For such applications phase 2 and phase 3 clinical trials have already started, using amongst others the anti-Aβ antibody solanezumab (DIAN-TU, NCT01760005) [83].

1.3. APP/PS1: a murine model for AD

Familial hereditary early onset AD (EOAD) is the result of mutations found on one or more of the main players in AD; such as APP and the various members of the γ-secretase complex, especially Presenilin 1 (PS1). The mutations that were found to lead to EOAD were not only pivotal in the basic research towards the etiology of AD, they also opened the path towards an impressive number of murine models for the disease. In this thesis one of the models, APPswe/PS1dE9 (APP/PS1), has been used as mouse model for Alzheimer's disease. The APP/PS1 model has been well characterized over the years and has proven to be a robust model for rapid amyloid aggregation, although no hyperphosphorylated tau will be observed along the amyloid pathology. Amyloid depositions in the APP/PS1 mice begin to develop around six months of age, with abundant plaques in the hippocampus and cortex by nine months, and continue to increase up to around 12 months of age [63,84]. The mice develop not only parenchymal plaques, but also a CAA-like phenotype, with amyloid depositions in cerebral and meningeal blood vessel walls [85,86]. Furthermore, the mice present severe astrocytosis in the vicinity of the plaques around six months of age and the number of GFAP-positive cells, a marker for activated astrocytes, increases progressively with age [87]. Abnormal behavior of the mice has been reported extensively, but the severity and timing of the behavioral deficits strongly depend on which specific test has been used [88]. Contextual memory, spatial learning, and spontaneous behavior, such as nest-building, have all been reported to be impaired, starting anywhere from six to 12 months of age [88–91]. Interestingly, the APP/PS1 mouse model has a relatively high incidence of spontaneous epileptic seizures, starting at a relatively young age, and presenting in approximately 55% of the mice at an age of 4.5 months [92]. Studies regarding the blood-brain barrier (BBB) integrity in the APP/PS1 model are contradicting, suggesting

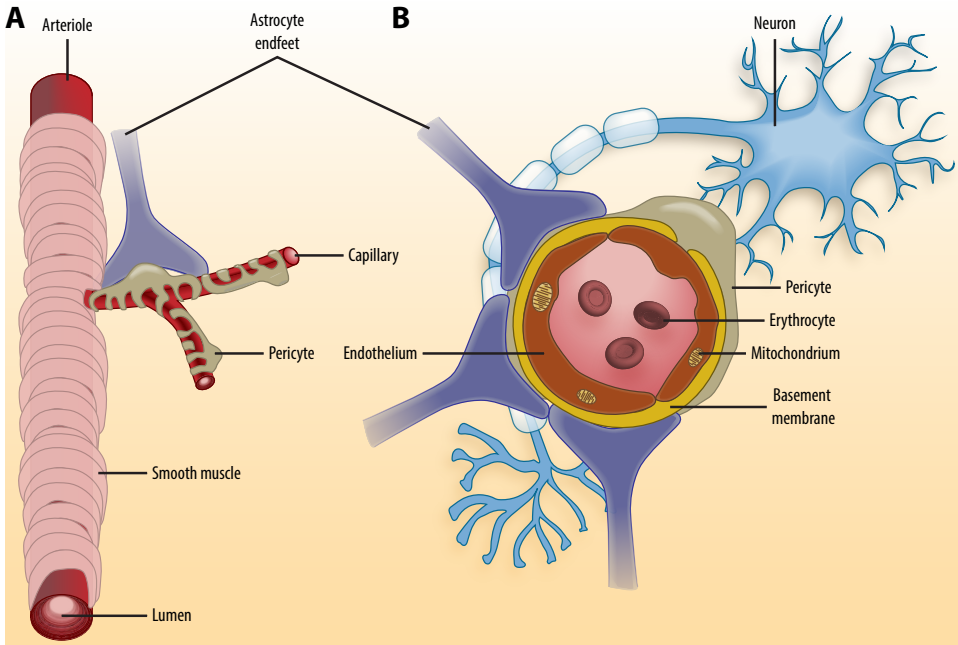

Figure 1.4. The blood brain barrier (BBB) is formed at the level of the capillary endothelium to separate the central nervous system from the bloodstream **(A)**. Pericytes and endothelial cells are separated for the most part by the basement membrane, save from a few points of contact through which the pericytes can communicate directly with the endothelium. Astrocyte endfeet cover the pericyte, basement membrane and endothelium, contributing to the tight regulation of the BBB. Together they form the neurovascular unit, supplemented by microglia (not depicted) and service one or more nearby neurons in the brain **(B)**. Figure modeled after data and figures from Zlokovic et al. [97] with permission by Cell Press and Hamilton et al. [98] under CC BY 4.0.

fundamental structural alterations at the interface between the brain and the blood, but often only minor permeability changes compared to wildtype [93–95].

1.4. The Blood-Brain Barrier

The BBB is the vascular barrier that separates the central nervous system (CNS) from the bloodstream and actively contributes to the maintenance of the crucial microenvironment of the brain parenchyma [96]. The BBB is formed by a tight network of highly polarized capillary endothelium, combined with pericytes, astrocyte endfeet, and the capillary basement membrane, Figure 1.4.

Tight and adherens junctions between adjacent endothelial cells close what normally would be the fenestræ; openings in the blood vessels that allow fast and efficient exchange of nutrients, macromolecules and circulating immune cells between the capillary bed and the organ [97]. Since there are no fenestræ at the blood-brain interface, permeability of the BBB is restricted to small lipid-soluble compounds with a molecular weight below 400 Da [99]. Larger compounds have to be actively transported; either via specialized influx and efflux transporters, or

via receptor- and adsorptive-mediated transcytosis [100]. The presence of the BBB is vital for the correct functioning of the brain; in virtually all human neurodegenerative disorders BBB disruption is evident to some degree, with corresponding changes in for example transporters and tight and adherens junctions [101], although it is hard to say whether BBB disruption or neurodegeneration starts first [97,102,103].

However, even an impaired BBB in an AD brain is still able to remove exogenous compounds, such as potential therapeutic drugs, out of the brain. This complicates the development of diagnostic, therapeutic and theragnostic compounds for neurodegenerative disorders significantly [104]. Any candidate, however promising during the developmental phase, will fail *in vivo* if brain delivery is needed but BBB passage not addressed. The development of *in vitro* blood-brain barrier models (for examples see references [105–108]) allowed for relatively high-throughput screening of possible compounds. One such a system, a static trans-well model with co-cultured astrocytes and bovine cerebral endothelial cells, was used to investigate the BBB passage propensity of a number of different llama derived heavy chain antibody fragments (VHH) directed against Aβ [109]. In these studies, the VHH showed promising *in vitro* BBB passage, depending strongly on the primary conformation of the N-terminus of the VHH. Unfortunately, this *in vitro* BBB passage observed for certain VHH could not be efficiently replicated *in vivo* in the APP/PS1 mouse model [85].

1.5. VHH: llama heavy chain only antibody fragments

A VHH is the variable domain of a heavy chain only antibody present in the blood of members of the camelid genus. In a serendipitous finding in 1989, a Belgian research group led by Dr. Hamers discovered seemingly incomplete circulating antibodies in the blood of camels. Next to the commonly known four-chain IgG antibodies, they found smaller antibodies composed only of one pair of heavy chain domains, lacking all light chain fragments [110]. In conventional heterodimeric IgG a heavy and a light chain variable domain (VH and VL, respectively) work together to form the antigen binding domain. The random association of the two domain repertoires allows for a great diversity of antigen recognition. However, in the llama heavy chain only antibodies (HCAbs), as there is no light chain present, the N-terminal domain of the single heavy chain forms this antigen recognition domain independently [111]. Even when this domain is completely isolated from the rest of the molecule, the isolated fragment is capable of binding antigens with affinities similar or exceeding those of conventional antibodies [112,113]. This isolated llama antibody fragment is called VHH and is, with a molecular size of 12-15 kDa, the smallest naturally occurring antigen-binding unit currently known, Figure 1.5 A and B [114].

Isolation of the approximately 600 base pair (bp) single gene fragment encoding the VHH revealed that the VHH is made up by four conserved framework (FR) regions surrounding three

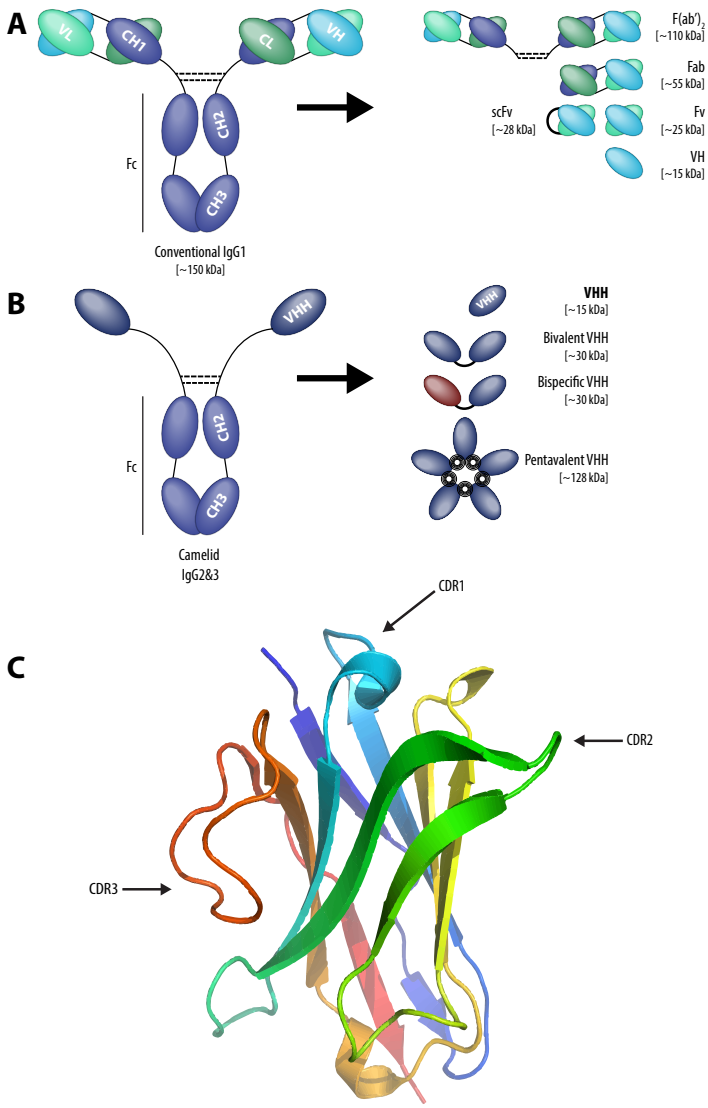

Figure 1.5. A variety of different antigen binding fragments can be derived from conventional **(A)** or heavy chain only **(B)** antibodies. The variable heavy chain, or antigen binding domain (VHH) of the camelid heavy chain only antibody is the smallest naturally occurring antigen-binding unit currently known. The VHH consists of four conserved frameworks separated by three complementary determining regions (CDR) **(C)**. The third CDR loop is elongated in VHH, compared to conventional heavy chain fragments, and attribute to the special characteristics of VHH. Part C is modeled after publically available work by S. Jähnichen on commons.wikimedia.org, based on crystal structure data from PDB (1I3V).

hypervariable (HV) regions. When the protein is folded, these HV regions form three loops that stick out of the antibody fragment and form the complementary-determining regions (CDR) of the VHH. The sequence within the loops is highly variable; while the length of loops 1 and 2 is quite restricted, loop 3 is significantly extended, especially in the dromedary derived VHH [115–117], Figure 1.5 C. This extended loop, stabilized by interloop disulfide bonds [118], allows

the VHH to enter hard-to-reach epitopes, such as the active sites of enzymes, which are often hidden for conventional antibodies [119–123]. As a result, VHH have become a very successful class of therapeutics, with applications ranging from interference with enzymatic active sites leading to neutralization of toxins [124–126], viruses [127,128], and fungi [129], blocking of apoptotic pathways in autoimmune diseases [130] and preventing aberrant protein aggregation [131], to binding spatially different Aβ depositions [75]. Other reported applications for VHH include early clinical diagnostics of rheumatoid arthritis, and affinity purification of conventional IgG [132,133].

Selection of the desired VHH is generally done through phage-display techniques [134,135]. In phage-display a fragment of interest, the VHH in this case, is expressed on the outside of a phage particle and is usually selected through various panning rounds of binding to exposed antigen epitopes [136,137]. Because the VHH is encoded by a single gene fragment of only 600 bp, genetic handling and modifications are relatively easily performed. As such, cloning of isolated VHH cDNA into phagemid vectors is rather straightforward and ensures easy construction of VHH-phage libraries. It has been done for naive [138,139], immunized [140,141] and (semi-)synthetic libraries [142,143]. The llama-derived VHH described in this thesis have been derived from both naive libraries (e.g. VHH-ni3A) and immunized libraries (e.g. VHH-pa2H). For the immunized libraries, llama immunization was performed using either vascular brain tissue from an HCHWA-D patient, supplemented with recombinant Aβ42 or alternatively, grey matter brain parenchyma from a Down syndrome patient with extensive plaque formation; strategies chosen to enrich the repertoire of amyloid-specific antibodies in the libraries [75,135,144]. As mentioned earlier, the obtained VHH showed remarkable Aβ recognition and some showed promising results in an *in vitro* BBB model, however, single tail-vein injections of the VHH in APP/PS1 mice failed to show sufficient delivery of the VHH into the brain [85,109].

1.6. Brain delivery of VHH

To develop VHH as useful tools to detect and fight neurodegeneration, efficient delivery into the brain is required. While VHH, at 12-15 kDa, are too big to passively diffuse over the BBB, various VHH have found to transmigrate over the BBB nonetheless. Specific and stringent selection yielded VHH that cross the BBB *in vivo* via receptor-mediated transcytosis, e.g. VHH FC_5 [145,146], but also other VHH have been found to cross the BBB either *in vitro* or *in vivo* [109,147]. However, not every VHH is able to cross the BBB, and slight changes in the amino acid composition of the antibody fragments may drastically change their crossing propensity [109]. In general, it seems that a high isoelectric point (pI) is favorable for BBB passage [148].

The small size of VHH results in very rapid clearance by the renal system [85]. When VHH pa-2H, selected against Aβ, is injected the APP/PS1 mouse model for AD, the VHH will show a

blood half-life of approximately 15-20 minutes [85] (and this thesis). The short blood half-life is also found for other peripherally injected VHH [149–152], and suggests that BBB passage can be hampered by the relatively short time that the VHH have to interact with the BBB *in vivo*. To increase the passage, and make VHH suitable for neurological applications, certain adaptations must be considered.

Examples of such adaptions can be found in research pertaining to brain delivery of other small molecules and proteins. They include direct cranial delivery via invasive injection methods, increasing blood half-life by prevention of renal clearance, and targeting the molecule to bind BBB transporters, and in the case of smaller proteins, gene therapy may be used as a delivery method. Direct cranial delivery has been performed via intracisternal injection [153], lumbar spinal injection [154,155], and direct topical application to exposed parenchyma [85], however, the procedure is often excessively invasive. Prevention of renal clearance can be obtained by increasing the size of the protein above the renal cut-off of (i.e. ≥65 kDa), e.g. via polymerization, PEGylation, or pentamerization [156,157], however, the method used to increase the protein in size may alter the characteristics of the protein itself, including physically impairing BBB passage due to its increased circumference. Continuous vascular infusion may help to keep blood levels of the protein at acceptable heights without altering the structure, but require significantly more amounts of the protein, production of which may not necessarily be practical or economically feasible [158,159]. To circumvent both alterations of the protein and unfavorable production requirements, the protein can be encapsulated in liposomes, which even can be targeted to the BBB directly. However, encapsulation has generally been limited to small, non-biological molecules and drug compounds [160–163].

In this thesis, four different methods of brain delivery of VHH have been explored: direct intracisternal injection, encapsulation in BBB targeted liposomes, fusion to a human IgG1-Fc-tail, and a gene therapy approach using Adeno Associated Virus (AAV).

1.7. Intracisternal injection of VHH to enter the brain

The method of direct intracisternal injection has been chosen to circumvent all delivery uncertainties, in order to proof the concept of functional VHH having a physiological effect in the murine brain. The method involves delivering a small amount (i.e. ≤ 10 µl at 7.5 mg/ml) of VHH into the cisterna magna, or fourth ventricle, via a percutaneous bolus injection. To follow its biodistribution in the living animals, the VHH was labeled with an infrared dye and the injected mice were imaged for up to two days in an *in vivo* optical small animal imaging system.

1.8. Liposomal encapsulation of VHH to target the BBB

The second method explored in this thesis is that of encapsulation of VHH in glutathione targeted liposomes, denoted G-technology [164,165]. The use of glutathione on the outside of liposomes targets the particle to the BBB, where the VHH carried inside the particle, will be delivered to the parenchyma. To follow the biodistribution of liposomal deliveries, conventionally the liposomes are radiolabeled, with for example the radioactive tracer Indium-111 (^{111}In) [166]. In the approach in this thesis, however, we opted to radiolabel the VHH itself, in order to truly determine the delivery of the internally carried payload into the brain. In order to increase shelf-life of the liposomal encapsulated VHH formulations, a novel radiolabeling protocol was developed to radiolabel the VHH after encapsulation.

1.9. IgG1-Fc fusion of VHH to prolong blood half life

To explore the effect of prolonged blood retention on the BBB passage of the VHH, the third delivery method involved the fusion of an Fc fragment to the VHH. In this method the VHH is complemented with the heavy chain crystallizable fragments 1 and 2 (CH_1 and CH_2) plus the hinge area, of a conventional human IgG1 [167]. By doing so, the reported rapid renal clearance could be prevented via two separate pathways. First of all, due to the Fc's inherent dimeric nature, the addition of an Fc-tail to the VHH causes the fusion protein to dimerize, increasing the size beyond the renal cut-off of 65 kDa (the dimeric VHH-Fc will become approximately 80 kDa in size). Secondly, presence of the Fc-tail allows interaction with the neonatal Fc receptor (FcRn), promoting recycling of the construct in the periphery [168–171]. The effect of Fc-fusion on BBB passage is a method that has not often been investigated for llama antibody fragments. Once again, biodistribution of the VHH-Fc construct was followed via In^{111} labeling.

1.10. Gene therapy to express VHH directly inside the brain

Finally, as VHH are transcribed from a single, 600 bp stretch of DNA, it is possible to employ genetic strategies to deliver the VHH at the site of interest. A DNA fragment of 600 bp easily fits in any viral vector, including the Adeno-Associated Virus (AAV) [172], which is known to efficiently transduce neurons after intracerebral injection, without eliciting an immunological response from either mouse models or human patients [173–175]. Depending on the packaging of the viral particle, antibody fragments can be expressed from virtually any desired location, both peripherally [176], and within the CNS [177,178]. By delivering the VHH as a gene-fragment directly to the neuronal cells in the APP/PS1 mice, a unique situation was created in which it was possible to follow the effect of the VHH on for example amyloid burden over a very long period of time, without the need for repeated injections or other interventions. To make optimal use of this situation, Emerald GFP (EmGFP: an exceedingly bright Green Fluorescent Protein)

was fused to the C-terminus of the VHH, allowing, in combination with intravital multi-photon microscopy through a cranial window, for the real-time longitudinal monitoring of expression, distribution and therapeutic efficacy of the VHH in living mice.

AIM AND OUTLINE OF THIS THESIS

This thesis is aimed at the development of VHH as therapeutic tools for neurodegenerative diseases with intracranial Aβ aggregation, such as AD, CAA and HCHWA-D, with a specific focus on the delivery of VHH to the brain.

Chapter 2 describes how the use of VHH can influence the production of Aβ in effort to reduce the amyloid burden in the brain. The chapter serves as a proof of principle that when the right VHH is applied at the correct location, the course of the disease might be modified. However, as safe, non-invasive and sustained delivery of compounds, including VHH, across the BBB is a hurdle not to be underestimated, the remainder of the thesis is focused on various methods of cranial delivery of VHH, in particular of VHH-pa2H. **Chapter 3** describes how encapsulation of VHH-pa2H in glutathione targeted liposomes significantly increases BBB penetration. **Chapter 4** shows that addition of a human IgG1-Fc tail to the VHH increases the time that the compound stays in the blood, but does not help to transfer the VHH across the BBB. Finally, in **chapter 5** the VHH is expressed directly in the brain. Here, AAV is used as a basis for viral gene therapy delivery. With the presented work in this thesis, a foundation is laid down for delivery of VHH to the brain.

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