Crossing barriers, delivery of llama antibody fragments into the brain

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CHAPTER

GENERAL DISCUSSION

In December 11, 2013, health ministers of the G8 stated the ambition to have a cure or a disease-modifying therapy for Alzheimer's disease (AD) available by 2025. An ambitious goal, which requires a tremendous world-wide, combined, effort, as currently not even a suggestion of either definitive cure or modifying therapy has yet been announced. In fact, the correct diagnosis of early stages of AD, accurate individualized prediction of disease progression, and even the exact etiology of the affliction are all very much elusive, yet essential for the fulfilment of the G8's ambition. Without it, AD remains the main cause of dementia and one of the great health-care challenges of the 21st century [1]. This thesis describes the potential use of llama antibody fragments (VHH) in an effort to detect, remove and prevent aggregates of the peptide amyloid beta (Aβ) in the brain in a pre-clinical research setting. Aggregation of Aβ is one of the hallmarks of AD and the focus of the majority of AD research, and it is mimicked in APPswe/PS1dE9 mice (APP/PS1), the model used throughout the thesis. We aim to answer two main questions: first, can we use VHH to alter Aβ aggregation and second, and equally important, how can we get the VHH into the brain. This thesis focusses on two VHH in particular: VHH-B3a, selected against the beta-secretase enzyme BACE1, and VHH-pa2H, selected against Aβ.

Anti-BACE1 VHH-B3a

In **chapter two**, a number of VHH are selected from an immune phage library derived from llama immunized with recombinant BACE1. The beta-secretase enzyme BACE1 is one of the enzymes that sequentially cleave the precursor protein APP into the peptide Aβ. Three VHH, VHH-B3a, -B1a, and -B5a, were analyzed and found to alter the activity of the secretase BACE1. VHH-B3a inhibits BACE1 activity, while VHH-B1a and -B5a both stimulate the enzyme's activity, each to a different extent. Inhibition of BACE1 has shown to be an efficient method to reduce not only brain Aβ levels, plaque burden and associated pathology in AD mouse models [2]. Furthermore, BACE1 inhibition has been able to reduce cognitive deficit such as in contextual fear conditioning, without leading to problems such as microhemorrhages, demyelination, neuromuscular dysfunction or other unexpected side-effects, even upon chronic treatment [3]. Naturally, these dramatic effects were observed in pre-clinical models in which the driver of the disease is over-expression of the human APP, often with mutations to further enhance preferential cleavage by BACE1 over the α-secretase complex. Nonetheless, a number of BACE1 modulating drugs are currently being investigated for clinical efficacy [4]. Although some of the studies were halted due to unexpected side-effects (e.g. BACE1 inhibitor LY2886721, NCT01561430, terminated by Eli Lilly and Company in 2014 due to abnormal liver enzyme elevations in 4 out of 70 patients [5]), other studies have progressed to phase 3 clinical trials (e.g. Verubecestat/MK-8931, NCT01953601 [6]). The results from these trials provide a promising outlook for BACE1 inhibiting VHH such as VHH-B3a [6-8].

Anti-Aβ VHH-pa2H

VHH-pa2H was selected from an immune phage library derived from llama immunized with brain parenchyma homogenates from a Down syndrome (DS) patient [9]. Due to the trisomy of chromosome 21, which carries the APP gene, DS patients tend to develop Aβ aggregates relatively early on in life [10]. VHH-pa2H has been selected for outstanding recognition against aggregates of Aβ, both post mortem in brain cryosections of humans and mice and *in vivo* in murine APP/PS1 brains [9,11]. Originally, over eight potential anti-Aβ VHH were characterized, including in addition to VHH-pa2H, the VHH-ni3A, -va2E, -vaE2, and pa11E. Throughout the thesis, we have used primarily VHH-pa2H as model VHH to develop and test new methods to get VHH into the brain. These new methods include liposomal delivery, elongation of blood half life via a human IgG1-Fc tail, and viral delivery. The main driving force behind the decision to select VHH-pa2H as model VHH over the other candidates was an early preliminary result showing acute disappearance of parenchymal plaques in APP/PS1 topically treated with the VHH; a study described in **chapter five**. Furthermore, VHH-pa2H was immediately available for efficient tag- and endotoxin-free production in yeast, which due to its scalability would

make this VHH a more favorable candidate for longitudinal *in vivo* studies compared to the other VHH.

VHH-B3a and –pa2H as AD therapeutics

One of the two main questions in this thesis is whether or not VHH can be employed as therapeutics for AD and related neurological disorders. As mentioned in the introduction of this thesis, VHH have been demonstrated to be highly applicable as therapeutics in general: inhibiting enzymatic active sites, preventing viral propagation, reducing aggregated proteins [12–19]. VHH-B3a described in **chapter two** does indeed inhibit the enzymatic activity of BACE1 *in vitro*, in a cellular based assay and *in vivo* in APP/PS1 mice, resulting in reduced Aβ concentrations in both plasma and brain. Longitudinal assays with VHH-pa2H, as described in **chapter five**, also seems to reduce the amyloid burden in mice, likely by interfering with the aggregation of Aβ, provided the VHH is present at a very early stage. However, despite the positive results, there are major caveats. Unlike the potent inhibition *in vitro* and in the cellular assay, the effect of VHH-B3a *in vivo* is rather low, and less consistent. This difference cannot be attributed to the addition of the dye IRD800CW, with which the VHH was tagged to facilitate monitoring of its *in vivo* presence. Also for VHH-pa2H, the *in vivo* effect was positive, yet largely inconsistent. Furthermore, VHH-pa2H seemed to have the most potential when delivered to the mice at the earliest possibility, directly after birth at P0. It must however be noted that in the *in vivo* longitudinal study with VHH-pa2H, the number of mice that could be included in the final analysis of amyloid burden is very low, and bears repetition to further increase significance and reduce the influence of individual variation. It is nonetheless clear that although VHH-pa2H is likely able to influence aggregation to some degree, it is not able to completely prevent or revert Aβ aggregation in the AD mouse model. Like the anti-BACE1 VHH-B3a, the anti-Aβ VHH-pa2H seems to have a restricted or limited influence on the hallmarks of AD. A minimal amount of Aβ might be crucial for neuronal cell survival and control of synaptic activity [20,21], so complete inhibition of Aβ production may not necessarily be desirable, yet it is unlikely that the currently observed effects proof to be sufficient for efficient treatment or prevention of AD. That being said, it does not take away the fact that the two VHH did have an effect. One of the VHH acted on the pathways before Aβ production and one after the Aβ peptide was already made. The finding provides evidence that it is possible to interfere with the pathway that leads up to Aβ production by selecting and utilizing the right VHH, and thus that the right VHH may indeed be able to alter the disease. Given the characteristics of VHH in general, and the positive effects of these VHH in particular, it is highly unlikely that no VHH can be selected, or created, that is able to influence the course of the disease more efficiently. To develop the VHH that is suitable for successful use as an AD therapeutic, one might however need to reconsider the entire chain of VHH selection.

VHH selection

VHH selection starts with the creation of a VHH cDNA library. VHH libraries are often created by immunizing llama or other members of the camelidae family with the antigen against which a VHH is desired. After development of an immune response, mRNA is isolated from lymphocytes, from which in turn a cDNA library of variable heavy chain domains, the VHH, is created [22–24]. The cDNA is then used to express the VHH as fusion protein with M13 bacteriophage coat proteins, allowing VHH to be presented by the phages, i.e. phage display. The phages can be selected by allowing the VHH to bind an immobilized antigen, wash away non-bound phages and then elute the bound phages from the antigen. These selected phages are then used to infect Escherichia coli, which are subsequently used to create a new, enriched, phage library. Multiple panning rounds of this selection procedure allows to enrich for a small number of highly favorable VHH, which then need to be identified as unique, e.g. via sanger sequencing, high resolution melting curve analysis, a combination of these methods, and characterized for their actual binding affinity [25]. Selected VHH can subsequently be cloned into vectors suitable for downstream applications, such as described in **chapters three, four** and **five** in this thesis. The whole process of immunization, library creation, VHH selection and characterization takes at least 3 to 4 months, and success is highly dependent on careful selection of the immunization antigen [26,27].

Although it is not strictly required to immunize the host camelid in order to obtain a diverse VHH library, it has been demonstrated that careful immunization with correctly folded antigen will determine the quality of the obtained VHH library and in turn the likelihood of selecting potent, usable VHH [26]. This effect is evident in the immune library that yielded VHH-pa2H, compared to the naive library from which VHH-ni3A was selected [9]. While the naive library-derived VHH-ni3A seems to have a preference for vascular Aβ depositions, it has become clear that VHH-pa2H has a much higher affinity for the aggregated peptide [9,11,27,28]. Specialized immunization programs may be able to create libraries naturally enriched for VHH that recognize epitopes limited to very specific variants of Aβ related dementias. This is especially relevant for variants of dementia caused by dominantly inherited mutations in APP. One example of such variant is Hereditary Cerebral Hemorrhages with Amyloidosis-Dutch type (HCHWA-D). HCHWA-D is a variant in which Aβ has a characteristic E22Q mutation (also known as E693Q in Aβ's precursor APP). As a result of the mutation, Aβ deposits heavily in the cerebral vasculature. It stands to reason that by immunizing a camelid with carefully isolated Aβ depositions that are found in afflicted cerebral vessels from a HCHWA-D patient, rather than recombinant Aβ-E22Q peptides, there will be a higher change to discover a VHH that binds HCHWA-D pathology with exceedingly high specificity and efficiency.

Further optimization can be achieved in the stringent selection of candidate VHH. As is seen

in **chapter two** of this thesis, the competitive elution of VHHs from inside or around enzymatic active sites by supplying increasing amounts of conventional enzyme specific ligands, can yield VHHs that are not only highly specific, but also able to modulate enzymatic activity, either in the inhibitory or excitatory direction [29].

Selection via phage display under application conditions is another way to improve the changes of enriching primarily for a blockbuster VHH [30]. In the case of HCHWA-D this may be selection on post mortem brain cryosections obtained from HCHWA-D patient material, or possibly in animal models that exhibit an HCHWA-D-like phenotype [31]. However, in view of screening throughput, both selection methods may only be feasible after initial panning rounds on classical phage-ELISA set-ups, where the antigen, here most likely recombinant Aβ-E22Q, is immobilized in a 96-wells plate.

However, these classical methods of VHH selection do not fully benefit from the advantages that the VHH can theoretically provide. While slightly outside the scope of this thesis, it is strongly advisable to modernize the process of VHH selection for scientific and therapeutic applications. The VHH has the advantage that it is a fully functional single domain antibody, made from one genetic fragment of around 600 basepairs, with three variable complementary determining regions (CDR) bordered by four highly conserved frameworks. Since there is no interplay between a heavy and a light chain as there is with conventional antibodies, mutations in the CDR, and especially the longer CDR3 loop, will primarily affect antigen recognition. As long as the mutations do not intervene with the folding or maturation of the VHH, randomization of these regions allows for the creation of a wide variety of VHH, able to bind a potentially unlimited array of antigens. Libraries containing these VHH with artificially randomized CDR loops are called synthetic phage display libraries. Synthetic phage display libraries are already routinely used to isolate VHH when their intended antigens cannot be used to induce an immune response in llamas or camels, e.g. when the antigen is highly toxic, pathogenic or non-immunogenic [32,33]. However, the libraries themselves are still very much random, selection of the one desired VHH out of these libraries is still done the conventional way, the eventually selected VHH may not be the most ideal VHH in the library, and on top of that, the theoretically most ideal VHH may not even be present in the randomly generated library. However, keeping the relatively simple lay-out of the genetic structure of VHH in mind, with the ever progressing technological advances in terms of sequencing of DNA, computing power, and massive database storage, it is technically possible to create a database in which the CDR diversity of known VHH is linked to their respective antigens, and subsequently used to predict, de novo and in silico, novel VHH sequences targeted towards very specific characteristics in terms of antigen recognition, enzyme interference, active transport mechanisms [22,34]. Selection of the VHH is the single most important step in developing the camelid antibody based therapeutic, diag-

nostic or theragnostic tool that will one day diagnose and prevent AD and related afflictions. To find the right VHH, if not by sheer serendipitous luck and a high number of iterations, the entire R&D chain surrounding VHH, from immunization, to selection, to identification, must be improved. The possibilities are endless and the rewards by far outweigh the investment, yet a major caveat needs to be overcome: it is currently are not even fully understood how the natural antibodies are able to create their impressive array of epitope recognition, let alone predict that of the relatively new VHHs. The fact that the interaction is now focused on only one domain with three or four CDRs to be elucidated, does make it promising, but the in silico prediction of epitope and paratope interaction is still in its infancy [35]

Blood brain barrier delivery

Once a VHH is selected and identified, the second main question in this thesis needs to be addressed: how can we get enough VHH effectively into the brain? This is not as straightforward as it may sound. The brain is the control center of the body. Together with the spinal cord it forms the central nervous system (CNS), and is arguably the single most important human organ. In order for the brain to work efficiently, even during severe bodily stress, it is crucial that the CNS maintains a strictly stable internal microenvironment; significantly more controlled than any other organ in the body. The CNS contains specific cell types not found in any other organ in the human body. In general it comes down to two categories of cells: neurons and glial cells [36]. While neurons are the cell-type that defines the brain, the neural specific supporting glial cells (astrocytes, oligodendrocytes, and microglia) as well as pericytes contribute to the local homeostasis and outnumber the neurons in certain areas of the brain, such as the cerebral cortex where the glia/neuron ratio equals approx. 3.72 [37–40]. To regulate and control the homeostasis in the CNS, specialized barriers strictly regulate both molecular influx and efflux at the interface between the CNS and the circulating blood. These barriers are either at the level of the cerebrospinal fluid (CSF) in the ventricles and the subdural space, forming the blood-CSF barrier (BCSFB), or at the level of the capillaries in the brain parenchyma, forming the blood-brain barrier (BBB) [41,42].

At the BBB tight 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. Together with pericytes, astrocytes and microglia, the capillary endothelium forms a modular neurovascular unit (NVU), each modular segment strictly regulating the microenvironment of a limited number of neurons (typically < 8) [43]. Passive exchange between the luminal and abluminal sides of the NVU is limited to diffusion of small non-polar solutes (< 600 Da in size),

Box 6.1. VHH Genetics

The family of Camelidae have three major subclasses of circulating γ immunoglobulins: IgG1, IgG2 and IgG3. IgG1 is a hetero tetrameric antibody (i.e. two heavy chains and two light chains) while IgG2 and IgG3 are homodimers of only heavy chains (i.e. two heavy chains, and no light chains) (Figure 6.1). The subtype IgG1 is composed by a variable domain that interacts with the antigen (VH) and three constant domains, named CH_1, CH_2 and CH_3, with a hinge region between CH_1 and CH_2. Directly after translation of the mRNA of the IgG1 heavy chain, the Endoplasmic Reticulum (ER) associated chaperon protein Heavy chain Binding Protein, BiP, binds the CH_1 domain. This CH_1-BiP interaction prevents folding of the domain, until BiP is replaced by a light chain. Should no light chain be present to replace BiP, the CH_1/BiP complex stays in the ER until it gets ubiquitinated via ubiquitin ligase U_3, exported to the cytoplasm and degraded by the proteasome [65]. If BiP is removed prematurely, the heavy chain domain folds into an unstable protein fragment, with hydrophobic amino acid side chains exposed to the intracellular environment, a situation that in humans leads to a variety of rare heavy chain diseases [66].

In the camelid subtypes IgG2 and IgG3, however, the CH_1 domain is not present [67]. This is the direct result of a G>A+1 point mutation in the 3' splice site recognition sequence between the CH_1 exon and the downstream intron [68]. The consensus, conserved among nearly all mammals, at the splice site between the 3' CH_1 exon and the 5' intron boundary (|) is TG|GT, but in the camel it is GG|AT [68]. Modification of the first invariant position (G+1) usually causes the skipping of the preceding exon [69,70]. Therefore, while it is highly homologous to other mammalian CH_1 domains, and would be perfectly in frame with the rest of the mRNA, the exon containing the camelid CH_1 domain for IgG2 and IgG3 gets spliced out and the resulting IgG heavy chain is devoid of the CH_1 domain. The skipped CH_1 exon does contain a Cys208Ser mutation that renders a crucial internal disulphide bridge impossible. It is thus possible that if the CH_1 exon would be translated, it may eventually still not be able to form a stable domain, which suggests that the exon skip was the only evolutionary option. Nonetheless, the Cys208Ser may also have been introduced after the silencing of the CH_1 exon occurred [68]. As a result of the absence of the CH_1 domain, the chaperone protein BiP cannot bind the heavy chain IgG and no light chain will be recruited before the heavy chain folds into its final, lowest free energy conformation and is released through the ER secretory pathways [71]. A 700-$Å^2$ hydrophobic area on the camelid VH domain, which is normally covered by the variable domain of the light chain (VL), contains several crucial mutations and non-destructive side-chain rotations to greatly reduce the hydrophobicity [67,72], which allows the folded heavy chain only antibody to remain functional and not succumb to the fate of heavy chain disease mentioned above. Once again, it is uncertain whether these mutations are an evolutionary driver or rather an evolutionary result of the CH_1 exon skip. Beside the evolutionary drive behind the genetic method, i.e. which genetic fact occurred first, the evolutionary reason for the general occurrence of heavy chain antibodies is an unsolved mystery, especially considering the co-occurrence of this type of antibody in various completely different animals (e.g. in camelidae [73] but also in cartilaginous fish [74–76]. In a review by Flajnik *et al.* three possible explanations have been offered: First it could have been used by a virus as a co-receptor for infection of B lymphocytes, causing light chain deletion. Second, light chains could be causative of amyloidosis, and thus its disappearance could be beneficial. And third, it could have arisen as a means to reach less accessible epitopes [75,77]. Regardless of the genetic evolutionary background, if one is to create a method for in silico, de novo VHH design, these crucial mutations, especially the ones in the formerly hydrophobic VH-VL interface area, are not to be meddled with.

all other compounds must follow either carrier-mediated influx (e.g. for glucose and amino acids), or receptor- or adsorptive mediated transcytosis (e.g. for transferrin and insulin) [42,44].

Since the VHH is, at on average 12-15 kDa, far larger than 600 Da, transport across the BBB must happen via active transport, i.e. specific receptor- or nonspecific absorptive-mediated trans-

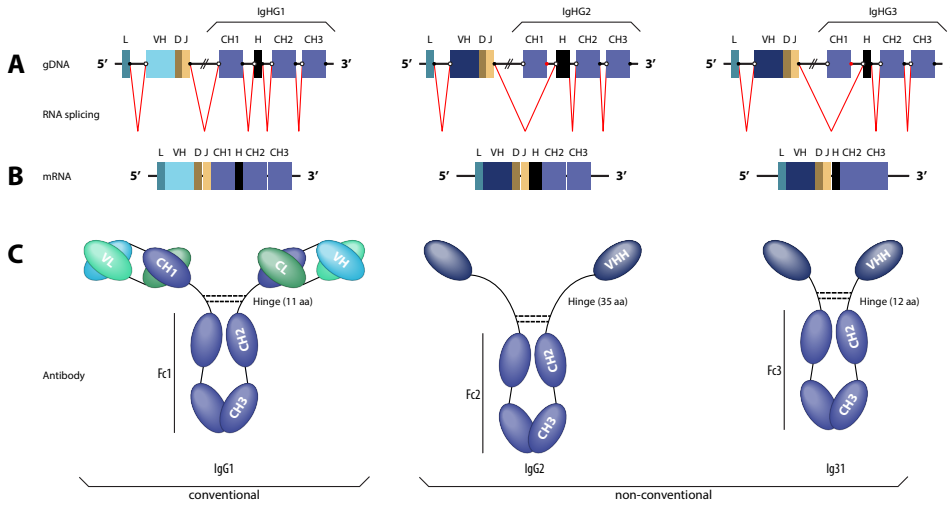

Figure 6.1. Members of the camelid family have three circulating IgG isoforms (**A**). Two of these – IgG2 and IgG3 – lack the CH1 domain in the messenger RNA (mRNA), due to an exon skip during splicing caused by a point mutation in the 3' splice site recognition sequence of that exon (**B**). Due to the lack of the CH1 domain, the light chain is not recruited to complement the IgG, and as a result, the final IgG2 and IgG3 antibodies are heavy chain only antibodies (**C**). Specific mutations in the variable domain of these IgGs prevent exposure of hydrophobic residues to the environment and allow IgG2 and IgG3 to function as complete, though non-conventional antibodies. **L**: leader sequence for sorting the protein into the correct secretory pathway; **D** and **J**: part of the Variable, Diversity and Joint region which allows for somatic recombination leading to the diversity of antibody repertoires; **H**: hinge region. The figure has been modelled after data and figures from IMGT®, the international ImMunoGeneTics information system® http://www.imgt.org (founder and director: Marie-Paule Lefranc, Montpellier, France) under permission of a general academic licence.

cytosis. This hypothesis is corroborated by the finding that VHH-ni3A when tested for *in vitro* BBB passage, could cross the *in vitro* BBB model only at 37°C and not at 4°C [28]. Furthermore, the finding that VHH with a high isoelectric point (pI) of around 9.4 were able to cross the BBB *in vivo*, while those that have a lower pI of around 7.7 do not, suggests that the presence of positive charges on the surface of the VHH, at physiological pH, are required for transcytosis [45]. This could be indicative that the transport of VHH is dependent on the negatively charged clathrin-coated pits, which are particularly enriched in the brain endothelium compared to peripheral endothelia and are the location to which cell surface receptors are generally recruited [46]. This is furthermore confirmed for a VHH specifically selected for *in vitro* and *in vivo* BBB passage, VHH-Fc5, which colocalizes with clathrin-coated pits and vesicles in human cerebral endothelial cells in culture [47]. It is not completely certain what receptor is responsible for the transcytosis of FC5, although over the years the α(2,3)-sialoglycoprotein receptor and the TMEM30A receptor have been put forward as the putative candidate by the same research group [47–49]. Evidence has solely been provided from *in vitro* research, and while VHH-FC5 has been used to shuttle other compounds effectively into the brain, currently no data is publically available showing the α(2,3)-sialoglycoprotein receptor, nor TMEM30A, as the BBB transporter of VHH *in vivo* [50]. It would be of monumental importance to determine, or confirm, which

receptor is required for the active transport of VHH across the BBB *in vivo*, with these receptors as primary candidates.

Although peripheral injection has reportedly led to cerebral uptake for some VHH, previous results for VHH-pa2H showed insufficient uptake *in vivo*, combined with a blood half-life of less than 20 minutes. The VHH gets rapidly cleared via the renal pathway [11]. There is no reason to assume the anti BACE1 VHH B3a will behave differently, especially given its low pI, see Table 6.1. To overcome this barrier, and to answer the second main question of this thesis, the studies described in this work explored various alternative delivery methods, i.e. intracerebral injection, liposomal delivery, elongation of the blood half-life and viral delivery.

Intracerebral injection

The BACE1 inhibitory VHH-B3a has a theoretical pI of 7.63, significantly lower than the recommended pI of 9.4 (Table 6.1), so it is not expected to cross the BBB via nonspecific adsorptive mediated transcytosis in the clathrin-coated pits and vesicles. Instead, to get VHH-B3a into the brain, an invasive injection straight into one of the cerebral ventricles was chosen. Although the method is technically challenging, correct injection leads to successful and widespread delivery, as was evident by both the *in vivo* and *ex vivo* imaging of the IRD800 labeled VHH (Figure 2.6 in **chapter two**) and the measured effect of the VHH on Aβ production (Figure 2.7). Efflux of the VHH was limited in so far that at least 24 hours after injection, the VHH was still measurable throughout the brain. However, for various reasons, intracerebral injections are not necessarily a sustainable method for long-term treatment of AD. For example, murine intracerebral injections are limited to 2- 10 µl volumes. As a result, to achieve a relevant concentration of VHH throughout the brain – which for VHH-B3a is approximately 1 µM – the concentration of VHH to be injected is necessarily very high, i.e. 500 µM, or approximately 6-7 mg/ml. Depending on the VHH in question, at such high concentrations the VHH may start to form aggregates and may lose its desired characteristics and may no longer be suitable for intracerebral injection. For regular applications, a therapeutic cerebral concentration of 1 µM may be difficult, if not impossible, to achieve. It is theoretically possible to lower the VHH concentration in the solution and repeat the injections multiple times to eventually achieve therapeutically relevant concentrations. However, the increased invasiveness of the treatment may be difficult to justify. Furthermore, depending on the actual efflux rate of the VHH from the brain into the periphery, it may not even be possible to sufficiently reduce the concentration of the injectable VHH, while staying within reasonable injection intervals. Delivering the VHH directly into the cerebral spinal fluid (CSF) via an outlet catheter from an implantable reservoir or pump may be preferred over repeated injections. However, CSF delivery is not necessarily the most ideal route. Although VHH-B3a was present throughout the brain, it may

Table 6.1. List of characteristics of studied VHHs. In general, most VHH have a theoretical pI around 8 to 10, an amino acid count between 120 and 130 and a molecular weight around 13 to 15 kDa. The addition of a hextahistadine (6his) and myc tag increases the size with a little over 2,5 kDa or 15-20 amino acids and lowers the pI slightly. The addition of a VSV and 6his tag has the same general effect, albeit a little less pronounced. The addition of the Fc tag and especially the addition of the EmGFP tag reduces the pI significantly, while the size of the VHH construct more than doubles. The theoretical pI was calculated using the ExPASy bioinformatics resource Portal program ProtParam, based on the guidelines described by Bjellqvist *et al.*[51].

VHH	Theoretical pI	AA	MW (kDa)
pa2H	9.86	120	13.000
pa2H-6his-myc	9.26	146	15.735
pa2H-Fc	8.96	351	38.856
pa2H-EmGFP	7.15	362	40.094
Htt7F4	5.29	130	14.058
Htt7F4-vsv-6his	6.57	147	16.202
Htt7F4-EmGFP	5.64	372	41.151
B1a	9.47	19	14.156
B3a	7.63	125	13.437
B5a	8.97	124	13.313
FC5	9.23	124	13.447
MCS-EmGFP	5.71	257	28.596
pa11E	9.56	120	12.884
pa11E-vsv-6his	9.47	137	15.029
pa11E-6his-myc	8.64	140	15.206
ni3A	9.56	126	13.834
ni3A-vsv-6his	9.46	143	15.978
va1G	7.80	148	15.621
pa4D	9.46	139	15.586
va2E	8.97	119	13.052
vaE2	8.62	126	13.895
ni8B	9.93	126	13.687

not necessarily have penetrated efficiently enough into the cortex [52]. CSF is found in the subarachnoid space, the ventricles and within the brain parenchyma in an area known as the Virchow Robin space, the area surrounding blood vessels that penetrate into the parenchyma. The Virchow Robin space gets obliterated, however, at the level of the capillary bed, restricting the CSF from reaching the deepest levels of the NVU [53]. As the cerebral vasculature has the largest, and most complete, interface with the rest of the brain (12-20 m2 in the average adult human brain), and the one allowing for the shortest diffusion distance to neurons, a peripheral approach would still be preferred over CSF delivery [42]. Therefore, **chapters three** and **four** aim at methods to deliver VHH-pa2H from the blood circulation into the brain.

Liposomal delivery

In **chapter three**, VHH-pa2H is encapsulated in GSH-PEG liposomes. GSH-PEG liposomes are glutathione-targeted and have previously been able to transfer small molecules across the BBB [54–56]. However, the use of GSH-PEG liposomes as encapsulating BBB-crossing carriers for bigger, functional proteins had not yet been demonstrated. In **chapter three** we show that it is possible to target VHH towards the murine brain using these liposomes. Once VHH-pa2H enters the parenchyma, it remains significantly longer in the brain of APP/PS1 mice compared to wildtype mice. The main difference between the APP/PS1 mice and the wildtype littermates is the presence of Aβ depositions in the former. It strongly suggests that VHH-pa2H is able to enter the brain and anchors onto the deposits if present, while it is rapidly removed again should the Aβ deposits not be there.

The rapid removal of VHH-pa2H from the wildtype brain is an interesting contrast with the observation found for the anti-BACE1 VHH-B3a, which stays around in the wildtype brain for at least 24 h, even though it does get slowly removed over time. A straightforward explanation could be that VHH-B3a binds ubiquitously expressed BACE1 on the cell surface of neurons, regardless of the presence of amyloid plaques. However, it is possible that the low pI of VHH-B3a hampers its interaction with the BBB transport receptor in the clathrin-coated pits on the abluminal side of the BBB, while VHH-pa2H is able to interact with the receptor and does experience efflux if not bound to plaques in the parenchyma. As VHH-pa2H, without any tags such as myc, vsv or hexahistadine, has a theoretical pI of around 9.86, while VHH-B3a has a theoretical pI of around 7.63, this explanation would match with the theory that the VHH must have a pI of above 9.4 to transfer across the BBB, albeit in efflux direction in this case. One way to address the question whether the lack of efflux is due to binding to ubiquitous BACE1 or due to another reason, is to inject VHH-B3a directly into the brains of BACE1 knockout animals as a control next to wildtype littermates. It is important to understand the mechanism behind the difference in observed efflux, as the rapid removal of nonbound VHH can be either beneficial or a hindrance depending on the intended use of the VHH. The rapid efflux of nonbound VHH-pa2H can be considered beneficial, especially if the VHH is to be employed as a marker for brain imaging, in which this effect will increase the signal-to-noise ratio of the VHH that is bound to the plaques [57]. However, when a prospective VHH must rather stay around in the brain, in order to continuously interfere with enzymatic activity, as is the case for VHH-B3a, then retention during reduced enzyme presence can be required.

Interestingly, only one of the two GSH-PEG liposomal formulations was able to shuttle VHH-pa2H into the brain, i.e. GSH-PEG EYPC and not GSH-PEG DMPC. This was observed despite the fact that the blood half-life for the GSH-PEG DMPC encapsulated VHH-pa2H was increased compared to the non-encapsulated VHH, albeit less than for the GSH-PEG EYPC li-

posomal formulation. The exact reason behind this difference was not clear. The two liposomal formulations differ slightly in chemical composition, but the size of the particles was highly similar. It is possible that the GSH-PEG DMPC liposomes are less stable than the GSH-PEG EYPC when loaded with protein fragments in an *in vivo* setting. It justifies optimizing the formulation of the liposomes for the *in vivo* use of encapsulated proteins such as VHH-pa2H, increasing *in vivo* stability and thereby possibly increasing the delivery of the antibody fragment into the brain even further. Another way to increase delivery might be to increase the concentration of the VHH inside the liposomes. In the experiments described in **chapter three**, VHH-pa2H had a maximum concentration of less than 0.4 mg per ml. While keeping in mind the objections against too heavily concentrated VHH solutions addressed before, 0.4 mg per ml is well below the critical concentration for VHH-pa2H, and increasing the pay-load of the liposomes will likely increase the yield of VHH crossing the BBB.

The use of the liposomes increases the retention of the VHH in the blood, but not necessarily the exposure time of the VHH to the BBB. It stands to reason that the increased signal in the blood over time comes solely from encapsulated VHH, and that as soon as the VHH is released from the liposome, the normal kinetics for non-tagged VHH come into play, yielding rapid renal clearance. The increased BBB transport is either the result of complete carriage of the liposome containing the VHH over the BBB, or the creation of a very local increased concentration of VHH at the BBB as the liposomes accumulate at the luminal side due to their glutathion targeting. For increased exposure of the VHH to the BBB, the VHH needs to be altered. For example, as described in **chapter four**, by the fusion of the VHH to the crystalizable fraction of the IgG tail, the Fc.

Fc-fusion

In **chapter four**, VHH-pa2H is fused to a human IgG1 Fc tail. Doing so increases the size of the VHH above the renal cut-off of 65 kDa, and additionally allows for recirculation by the neonatal Fc receptor (FcRn), both characteristics aimed to increase the blood half life of the VHH. By increasing the blood half life, the VHH has, theoretically, more time to interact at the BBB, and thus more time to engage in active transport into the brain. Indeed, 48 hours after intravenous injection, the amount of VHH-pa2H-Fc fusion protein in the blood was significantly higher in both APP/PS1 and wildtype mice compared to non Fc-fused VHH-pa2H. However, brain uptake of the fusion VHH was not higher in the transgenic mice compared to the wildtypes. In **chapter three** it was already determined that once the VHH gets into the brain, it will bind amyloid depositions if present. The fusion of the Fc tail to VHH-pa2H did not hamper its ability to bind amyloid depositions in cryosections, and therefore there is no reason to assume that the fusion VHH would not bind the amyloid plaques in the brains of the APP/PS1 mice if it would

have gotten past the BBB. It is therefore clear that VHH-pa2H-Fc, despite the increased time that it has spend in the circulation, did not fully cross the BBB into the murine brain. There are multiple possible explanations for this observation. First and foremost, the addition of the Fc tail may have reduced the propensity of the VHH to cross the BBB in itself. The theoretical pI of VHH-pa2H, already not ideal, becomes even lower due to the addition of the tail (see Table 6.1). Furthermore, the increased molecular weight of the construct, on one hand the necessary trick to keep the VHH longer in the bloodstream, may on the other hand be a cause for steric hindrance, preventing adequate interaction with whichever transporter is normally recruited to shuttle the VHH into the brain. And finally, the interaction of the VHH-Fc fusion protein with the FcRn not only recycles the construct in the periphery, it may also be responsible for the directed efflux of IgG from the brain parenchyma into the blood, which may mean that transported VHH immediately gets removed, even before it reaches the extracellular Aβ depositions [58–60]. Secondly, despite the increased presence of the VHH in the blood and thus its increased presentation to the BBB, VHH-pa2H itself may not be the ideal candidate to cross the barrier from the blood into the brain, regardless of its tail. A study by Farrington *et al.* showed how the fusion of an Fc tail to their VHH-FC5 yielded similarly increased blood retention and reported that the fusion construct was able to drag more of an analgesic opioid peptide into the brain compared to controls, as was evident in an attenuated pain response in male Wistar rats. However, it must be noted that the concentration of VHH-FC5 used in that study was significantly higher than what was used for VHH-pa2H-Fc. Increasing the amount of VHH-pa2H-Fc to 25 mg/kg per injection may help to increase BBB passage. But, to increase the presence of VHH-pa2H in the parenchyma beyond any doubt a third option was explored, which takes full advantage of the small size and incredible stability and foldability of the single domain antibody fragment, delivery via adeno associated virus (AAV) directly inside the brain.

Viral delivery

In **chapter five**, the genetic code of VHH-pa2H is delivered to the neuronal cells via means of adeno associated virus (AAV). The transduced cells in the brain subsequently produce the VHH themselves, continuously replenishing any effluxed VHH. A C-terminally fused Emerald Green Fluorescent Protein (EmGFP) allowed for real-time *in vivo* monitoring of the longitudinal effect of the VHH-EmGFP construct through a permanent cranial window. The results from the longitudinal study suggests that the tested VHH-pa2H may have preventive characteristics against Aβ accumulation. However, acute effects on pre-existing Aβ burden was not observed.

AAV is ideally suited for the *in vivo* delivery of VHH constructs. The small size of the VHH, at less than 600 bp, stays well within the packaging limits of AAV, i.e. 4.7 kb between the Inverted Terminal Repeat elements.

There were some interesting observations in this study. First of all, VHH-pa2H-EmGFP was actively expressed in and secreted from standard HEK293T cells in cell culture. The secreted fusion protein retained both its high specificity for Aβ depositions provided by VHH-pa2H and the bright fluorescence of the EmGFP. As a result, brief exposure to cultured medium from the cells was sufficient to label amyloid plaques and CAA in both human and murine brain cryosections with the fluorescent EmGFP visible under both a standard fluorescent microscope as well as under the multiphoton microscope. Not only did this reduce the duration of the protocol for staining AD/CAA positive material from multiple days down to a few hours, it also provides any research lab that has access to a standard cell culture set-up, with an affordable in-house and on demand, inexhaustible production line for anti-Aβ immunofluorescence. Furthermore, there is no reason to expect that this procedure of functionally secreted VHH-EmGFP cannot be translated to other VHH and other fluorescent biomolecules.

Secondly, the VHH-EmGFP delivered as AAV constructs directly after birth, were not expressed within the first three weeks after injection. This observation of delayed onset of ubiquitous expression within transduced cells was also observed in the cell culture. This delay hampered viability of *in vitro* cellular studies, as cells reach full confluency before a signal is detected. However, for the purpose of continuous and sustained delivery of VHH to the parenchyma in the *in vivo* settings, the delay is not an issue. In fact, in the case of the mice which received a cranial window to observe the VHH expression and its effect on amyloid depositions in real time, the delay allowed for ample time to install the window and allow the animals some time to recover before the first imaging session was to take place.

Expression of the VHH-pa2H-EmGFP construct within neuronal cells in the mice that were injected at five months of age and sacrificed up to seven months later was no longer detected. This was in stark contrast with the mice that were injected as adults and sacrificed up to three months later. Nonetheless, while VHH-pa2H-EmGFP was no longer visibly produced, it was very visibly present on the Aβ depositions in the parenchyma and around the vasculature in the "five months old mice". This observation once more reiterates the potency of VHH-pa2H to bind amyloid plaques. Unfortunately, it also shows that VHH-pa2H may not be able to remove amyloid plaques once they are formed.

In recent years pre-clinical and clinical research into gene-therapy as treatment of genetically inherited diseases, certain types of cancer, Parkinson's disease and even HIV has grown exponentially [61–64]. VHH can play a major role in this development, as demonstrated in **chapter five** of this thesis.

Side effects of VHH alterations

Whether the addition of fusion proteins or chelators affects the affinity or the function of the VHH is something that needs to be examined in much greater detail. As seen in this thesis, creating a VHH-EmGFP allows for single step immunofluorescence on cryosections and allows for efficient *in vivo* monitoring. The addition of the Fc tail to the VHH immediately led to a significant increased retention of the VHH in the periphery of the mice. However, it is crucial to determine the effect of the fused protein on the physiological functions of the VHH. For example, it is interesting to note that the recognition pattern for VHH-pa2H-Fc and VHH-pa2H-EmGFP are very similar but not exactly the same. It may have to do with the brightness of EmGFP, or the need for secondary antibodies when using VHH-pa2H-Fc, but even when these factors are taken into account the Fc fused VHH seems to recognize a broader pattern around the parenchymal plaques compared to the more dense pattern obtained by the EmGFP fusion construct. The fact that the control VHH, VHH-Htt7F4, in both cases does not associate with the amyloid depositions, suggests that the binding of VHH-pa2H is specific; it is not a random sticky association of the fusion proteins to the amyloidosis as a result of any addition to any VHH. Neither do the additions create nonspecific association to other structures in the brain, such as the non-amyloidogenic vasculature, as none of the anti-Aβ VHH fused to either Fc or EmGFP show any binding on control cryosections derived from healthy age-matched donors. Therefore, the different patterns between VHH-pa2H-Fc and ss-VHH-pa2H-EmGFP must be driven by the effect that the fusion moiety has on the binding specificity of the VHH itself. Whether it is caused by the steric difference between the VHH and its fusion protein, e.g. EmGFP is twice as big as the VHH itself, or the difference in pI or other molecular effects, is a topic that has not been explored in this thesis, but warrants further investigation, especially to solidify the use of VHH as mainstream alternatives for current conventional antibody applications.

However, from a practical point of view, to determine to what extent this effect alters the function of the VHH is ultimately relevant in particular to the already selected and accepted VHH. Merely to understand and accept that the addition of, for example, EmGFP may alter the function of VHH, is sufficient if one changes the way the VHH are selected. The current generation of VHH has been selected by looking for a VHH that binds immobilized Aβ and once that had been done, we started to look what else we could do with them. Yet, if one is to ultimately apply the VHH in combination with a fusion protein, such as EmGFP, it would make more sense to display not a VHH, but a VHH-EmGFP to the antigen of interest. Now that it is known that the VHH can easily maintain such an addition, there is no reason not to create a library in which the pUC5071 phagemid vector is updated to pUC5072: where EmGFP is automatically inserted C-terminally to the VHH, or pUC5073: VHH-Fc. Once more, all these factors can still be taken into account in the envisioned in silico design of VHH.

Outlook for VHH

VHH are the antibodies of the future and the future is now. The antibody market, especially for therapeutical purposes, is rapidly expanding. Monoclonal antibodies dominate the top-10 billion dollar generating blockbuster drug list, expecting to grow in market value from 150 billion USD in 2011 to over 250 billion USD in 2017 [78]. Meanwhile, VHH have all the benefits of conventional antibodies, complemented with the intrinsic characteristics of the protruding CDR3 loop. At a practical, academic level, they are easily produced in standard laboratory settings, providing an unlimited supply of fresh, ready-to-use antibody compound for standard characterization of proteins for which otherwise expensive full size antibodies need to be obtained. Sharing VHH among academics can be as convenient as maintaining a public database. Researchers can then simply synthesize a 600 bp dna fragment, a commercial outsourceable venture that can cost less than 150 USD in 2016, which is less than most off-the-shelf conventional antibodies. It even allows researchers to create their own VHH, add their own tags, be it tags to purify like 6-HIS or Flag or tags to detect, like myc, V5, or fluorophores like EmGFP or mCherry. Furthermore, it is then possible to easily combine the VHH into biheads or pentamers with VHH that have different, complementary characteristics. The commercialization of VHH selection and production is expected to rapidly increase in the coming years, as many of the patents associated with heavy-chain only antibodies, mostly in the hands of the biopharmaceutical company Ablynx, will expire in Europe and the USA between 2013 and 2017 [79].

The versatility of the antibody fragments, as demonstrated in this thesis, is unmatched by conventional IgGs. The only downside of many selected VHH is their difficulty to recognize linearized epitopes in applications such as Western blot. However, some VHH are able to recognize antigens presented on Western blot membranes (M. Schut, unpublished data) and the generally observed reluctance to bind linearized epitopes is possibly a result of the method of selection, which usually involves immobilized, correctly folded antigens, in phage display. When one would like to enrich for VHH that are able to recognize linearized epitopes, selection procedures can be adjusted accordingly and there is no reason to expect that no suitable VHH can be found. As stated repeatedly before, improved use of *in silico* and *de novo* VHH selection can be of use in this regard.

Within the field of dementia research, even specifically within AD research, a wealth of opportunities await the further development of the llama antibody fragments. The VHH discussed throughout the majority of this thesis, VHH-pa2H, has been focused on the removal of existing amyloid beta peptides, either while forming or after forming amyloid plaques. However, AD may be fought in a plethora of different ways. As discussed and touched upon in **chapter two**, a therapy may aim to interfere in the production of the Aβ peptide itself by addressing the

cleavage of the precursor protein by BACE1 secretase, forcing the processing of APP into the non-amyloidogenic pathway.

Alternatively, one could address the initial presentation of APP to the secretases, rather than interfering with their overall function. Secretase activity is not evenly distributed throughout neurons, as each of the involved enzymes is a target of highly specific polarized sorting events. Furthermore, APP trafficking throughout the neuronal cells is mediated by sorting receptors such as Sortilin and SorLA [80]. SorLA-APP interaction prevents APP from routing into late endosomes where proteolytic cleaves occurs [81–83]. As a result, less APP will be cleaved into Aβ, leading to a reduced amyloid phenotype in mice [84–86]. Developing a VHH that is able to stimulate this process, similarly to the stimulatory effect observed for VHH-B1a and VHH-B5a, may help to reduce the presence of Aβ by reducing the processing of APP altogether. As less APP will be cleaved, it automatically means less sAPPα will be created as well. What the effect of this reduction will be on the brain and the rest of the body, will need to be elucidated [87]. However, it is a distinct possibility that the overly abundant presence of Aβ is rather a symptom than a cause of AD, especially for the sporadic variant, and a VHH can possibly be developed that addresses the root cause of the problem, once elucidated.

Finally, as discussed before, VHH can be employed not only as therapeutic entities, but also as diagnostic tools. The development of the fluorescently tagged VHH-EmGFP may help in this regard, but for this aspect both BBB passage and *in vivo* fluorescent detection in humans needs to be improved. Radioactively labeled VHH, such as used in **chapter three**, can be a better alternative, but even then one most keep in mind that detecting absolute Aβ burden may not be the ultimate way to diagnose AD and especially AD progression, but merely establish whether an individual is Aβ positive [88,89]. However, the application of VHH is not limited to the murine AD brain; the theoretical possibilities are endless. In fluorescence guided surgery, one can employ a VHH-EmGFP tagged with DTPA-In^{111} to be used in the same way as various similar hybrid tracers are currently used for sentinel node detection [90]. However, like conventional antibodies, the VHH can be selected for any targetable epitope, while, unlike conventional antibodies, remain within reasonable production costs even at the high doses necessary to allow for intraoperative imaging of the fluorescent moiety. In any diagnostics one can create a chip where you spot your target, such as a drop of patient blood or CSF, a skin or muscle biopsy, and subsequently flow an array of differently fluorescent VHH fusions, e.g. VHH-EmGFP, VHH-YFP, VHH-mCherry, and read-out the final fluorescent spectrum to obtain immediate, single-step, quantitative diagnostic read-outs, akin to a modified ELISA [91–93]. The stability of VHH may make them suitable for harsh conditions such as in (tropical) field hospitals, which can be crucial for applications such as the detection of mosquito-borne diseases like malaria, Zika, and dengue, other viruses and parasites.

Overall conclusion.

The two main questions in this thesis are whether the VHH can be used as an anti-AD therapeutic and how do we get the VHH into the brain most efficiently. VHH-pa2H and VHH-B3a do have the potential to be used as therapeutic, perhaps in combination therapy, but are not sufficiently potent by themselves to revert or inhibit AD progression once the disease has a foothold in the brain. However, VHH in general should be able to be developed into an efficient therapeutic, but the ideal VHH still needs to be found, or designed. For this, in silico VHH design should receive more attention in future years. To get the VHH into the brain, gene-therapy seems to be the easiest and most efficient route, fully utilizing the advantage that VHH have over other antibody approaches, namely the short, single domain, antigen interacting fragment, translated from a single gene fragment, which, even with the fusion to a large fluorescent protein or other tag, can still be delivered via the small, easy to create and deploy, adeno associated virus.

REFERENCES

1. Scheltens, P., Blennow, K., Breteler, M. M. B., de Strooper, B., Frisoni, G. B., Salloway, S. & Van der Flier, W. M. Alzheimer's disease. Lancet (London, England) 388(10043):505–17 (2016).
2. Ghosh, A. K., Brindisi, M. & Tang, J. Developing β-secretase inhibitors for treatment of Alzheimer's disease. J. Neurochem. 120 Suppl(SUPPL. 1):71–83 (2012).
3. Thakker, D. R., Sankaranarayanan, S., Weatherspoon, M. R., Harrison, J., Pierdomenico, M., Heisel, J. M., Thompson, L. a., Haskell, R., Grace, J. E., Taylor, S. J., et al. Centrally Delivered BACE1 Inhibitor Activates Microglia, and Reverses Amyloid Pathology and Cognitive Deficit in Aged Tg2576 Mice. J. Neurosci. 35(17):6931–6936 (2015).
4. Vassar, R. BACE1 inhibitor drugs in clinical trials for Alzheimer's disease. Alzheimers. Res. Ther. 6(9):1–14 (2014).
5. May, P. C., Willis, B. A., Lowe, S. L., Dean, R. A., Monk, S. A., Cocke, P. J., Audia, J. E., Boggs, L. N., Borders, A. R., Brier, R. A., et al. The potent BACE1 inhibitor LY2886721 elicits robust central Aβ pharmacodynamic responses in mice, dogs, and humans. J. Neurosci. 35(3):1199–210 (2015).
6. Yan, R. Stepping closer to treating Alzheimer's disease patients with BACE1 inhibitor drugs. Transl. Neurodegener. 5:13 (2016).
7. Evin, G. Future Therapeutics in Alzheimer's Disease: Development Status of BACE Inhibitors. BioDrugs 30(3):173–94 (2016).
8. Panza, F., Seripa, D., Solfrizzi, V., Imbimbo, B. P., Lozupone, M., Leo, A., Sardone, R., Gagliardi, G., Lofano, L., Creanza, B. C., et al. Emerging drugs to reduce abnormal β-amyloid protein in Alzheimer's disease patients. Expert Opin. Emerg. Drugs :1–15 (2016). doi:10.1080/14728214.2016.1241232
9. Rutgers, K. S., van Remoortere, A., van Buchem, M. A., Verrips, C. T., Greenberg, S. M., Bacskai, B. J., Frosch, M. P., van Duinen, S. G., Maat-Schieman, M. L. & Van der Maarel, S. M. Differential recognition of vascular and parenchymal beta amyloid deposition. Neurobiol. Aging 32(10):1774–1783 (2009).
10. Head, E., Lott, I. T., Wilcock, D. M. & Lemere, C. A. Aging in Down Syndrome and the Development of Alzheimer's Disease Neuropathology. Curr. Alzheimer Res. 13(1):18–29 (2016).
11. Nabuurs, R. J. A., Rutgers, K. S., Welling, M. M., Metaxas, A., de Backer, M. E., Rotman, M., Bacskai, B. J., van Buchem, M. A., van der Maarel, S. M. & van der Weerd, L. In vivo detection of amyloid-β deposits using heavy chain antibody fragments in a transgenic mouse model for Alzheimer's disease. PLoS One 7(6):e38284 (2012).
12. Yardehnavi, N., Behdani, M., Pooshang Bagheri, K., Mahmoodzadeh, A., Khanahmad, H., Shahbazzadeh, D., Habibi-Anbouhi, M., Ghassabeh, G. H. & Muyldermans, S. A camelid antibody candidate for development of a therapeutic agent against Hemiscorpius lepturus envenomation. FASEB J. 28(9):4004–14 (2014).
13. Hmila, I., Abdallah R, B. A. Ben, Saerens, D., Benlasfar, Z., Conrath, K., Ayeb, M. El, Muyldermans, S. & Bouhaouala-Zahar, B. VHH, bivalent domains and chimeric Heavy chain-only antibodies with high neutralizing efficacy for scorpion toxin AahI'. Mol. Immunol. 45(14):3847–3856 (2008).
14. Gad, W., Ben-Abderrazek, R., Wahni, K., Vertommen, D., Muyldermans, S., Bouhaouala-Zahar, B. & Messens, J. Wheat germ in vitro translation to produce one of the most toxic sodium channel specific toxins. Biosci. Rep. 34(4) (2014).
15. Cardoso, F. M., Ibañez, L. I., Van den Hoecke, S., De Baets, S., Smet, A., Roose, K., Schepens, B., Descamps, F. J., Fiers, W., Muyldermans, S., et al. Single-domain antibodies targeting neuraminidase protect against an H5N1 influenza virus challenge. J. Virol. 88(15):8278–96 (2014).
16. Garaicoechea, L., Aguilar, A., Parra, G. I., Bok, M., Sosnovtsev, S. V, Canziani, G., Green, K. Y., Bok, K. & Parreño, V. Llama nanoantibodies with therapeutic potential against human norovirus diarrhea. PLoS One 10(8):e0133665 (2015).

17. Dolk, E., van der Vaart, M., Lutje Hulsik, D., Vriend, G., de Haard, H., Spinelli, S., Cambillau, C., Frenken, L. & Verrips, T. Isolation of llama antibody fragments for prevention of dandruff by phage display in shampoo. Appl. Environ. Microbiol. 71(1):442–50 (2005).

18. Scheuplein, F., Rissiek, B., Driver, J. P., Chen, Y.-G., Koch-Nolte, F. & Serreze, D. V. A recombinant heavy chain antibody approach blocks ART2 mediated deletion of an iNKT cell population that upon activation inhibits autoimmune diabetes. J. Autoimmun. 34(2):145–54 (2010).

19. Impagliazzo, A., Tepper, A. W., Verrips, T. C., Ubbink, M. & van der Maarel, S. M. Structural basis for a PABPN1 aggregation-preventing antibody fragment in OPMD. FEBS Lett. 584(8):1558–64 (2010).

20. Plant, L. D., Boyle, J. P., Smith, I. F., Peers, C. & Pearson, H. a. The production of amyloid beta peptide is a critical requirement for the viability of central neurons. J. Neurosci. 23(13):5531–5535 (2003).

21. Pearson, H. A. & Peers, C. Physiological roles for amyloid beta peptides. J. Physiol. 575(Pt 1):5–10 (2006).

22. Dmitriev, O. Y., Lutsenko, S. & Muyldermans, S. Nanobodies as Probes for Protein Dynamics in Vitro and in Cells. J. Biol. Chem. 291(8):3767–75 (2016).

23. Ignatovich, O., Jespers, L., Tomlinson, I. M. & de Wildt, R. M. T. Creation of the large and highly functional synthetic repertoire of human VH and Vκ domain antibodies. Methods Mol. Biol. 911:39–63 (2012).

24. Olichon, A. & de Marco, A. Preparation of a naïve library of camelid single domain antibodies. Methods Mol. Biol. 911:65–78 (2012).

25. Pepers, B. A., Schut, M. H., Vossen, R. H., van Ommen, G.-J. B., den Dunnen, J. T. & van Roon-Mom, W. M. Cost-effective HRMA pre-sequence typing of clone libraries; application to phage display selection. BMC Biotechnol. 9(1):50 (2009).

26. Pardon, E., Laeremans, T., Triest, S., Rasmussen, S. G. F., Wohlkönig, A., Ruf, A., Muyldermans, S., Hol, W. G. J., Kobilka, B. K. & Steyaert, J. A general protocol for the generation of Nanobodies for structural biology. Nat. Protoc. 9(3):674–93 (2014).

27. Klooster, R., Rutgers, K. S. & van der Maarel, S. M. Selection of VHH antibody fragments that recognize different Aβ depositions using complex immune libraries. Methods Mol. Biol. 911:241–53 (2012).

28. Rutgers, K. S., Nabuurs, R. J. A., van den Berg, S. A. A., Schenk, G. J., Rotman, M., Verrips, C. T., van Duinen, S. G., Maat-Schieman, M. L., van Buchem, M. A., de Boer, A. G., et al. Transmigration of beta amyloid specific heavy chain antibody fragments across the in vitro blood-brain barrier. Neuroscience 190:37–42 (2011).

29. Dorresteijn, B., Rotman, M., Faber, D., Schravesande, R., Suidgeest, E., van der Weerd, L., van der Maarel, S. M., Verrips, C. T. & El Khattabi, M. Camelid heavy chain only antibody fragment domain against β-site of amyloid precursor protein cleaving enzyme 1 inhibits β-secretase activity in vitro and in vivo. FEBS J. 282(18):3618–3631 (2015).

30. Dolk, E., Verrips, T. & de Haard, H. Selection of VHHs under application conditions. Methods Mol. Biol. 911:199–209 (2012).

31. Herzig, M. C., Eisele, Y. S., Staufenbiel, M. & Jucker, M. E22Q-mutant Abeta peptide (AbetaDutch) increases vascular but reduces parenchymal Abeta deposition. Am. J. Pathol. 174(3):722–6 (2009).

32. Yan, J., Li, G., Hu, Y., Ou, W. & Wan, Y. Construction of a synthetic phage-displayed Nanobody library with CDR3 regions randomized by trinucleotide cassettes for diagnostic applications. J. Transl. Med. 12(1):343 (2014).

33. Sabir, J. S. M., Atef, A., El-Domyati, F. M., Edris, S., Hajrah, N., Alzohairy, A. M. & Bahieldin, A. Construction of naïve camelids VHH repertoire in phage display-based library. C. R. Biol. 337(4):244–9 (2014).

34. Rutten, L., de Haard, H. & Verrips, T. Improvement of proteolytic stability through in silico engineering. Methods Mol. Biol. 911:373–81 (2012).

35. Peng, H.-P., Lee, K. H., Jian, J.-W. & Yang, A.-S. Origins of specificity and affinity in antibody-protein interactions. Proc. Natl. Acad. Sci. U. S. A. 111(26):E2656-65 (2014).

36. Bergmann, C. C., Lane, T. E. & Stohlman, S. A. Coronavirus infection of the central nervous system: host-virus stand-off. Nat. Rev. Microbiol. 4(2):121–32 (2006).

37. Azevedo, F. A. C., Carvalho, L. R. B., Grinberg, L. T., Farfel, J. M., Ferretti, R. E. L., Leite, R. E. P., Jacob Filho, W., Lent, R. & Herculano-Houzel, S. Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. J. Comp. Neurol. 513(5):532–41 (2009).

38. Herculano-Houzel, S. The glia/neuron ratio: How it varies uniformly across brain structures and species and what that means for brain physiology and evolution. Glia 62(9):1377–1391 (2014).

39. Han, X., Chen, M., Wang, F., Windrem, M., Wang, S., Shanz, S., Xu, Q., Oberheim, N. A., Bekar, L., Betstadt, S., et al. Forebrain engraftment by human glial progenitor cells enhances synaptic plasticity and learning in adult mice. Cell Stem Cell 12(3):342–53 (2013).

40. Prinz, M. & Priller, J. Microglia and brain macrophages in the molecular age: from origin to neuropsychiatric disease. Nat. Rev. Neurosci. 15(5):300–12 (2014).

41. Johanson, C. E., Stopa, E. G. & McMillan, P. N. The blood-cerebrospinal fluid barrier: structure and functional significance. Methods Mol. Biol. 686:101–31 (2011).

42. Abbott, N. J. Blood-brain barrier structure and function and the challenges for CNS drug delivery. J. Inherit. Metab. Dis. 36(3):437–49 (2013).

43. Iadecola, C. & Nedergaard, M. Glial regulation of the cerebral microvasculature. Nat. Neurosci. 10(11):1369–76 (2007).

44. Abbott, N. J., Patabendige, A. a K., Dolman, D. E. M., Yusof, S. R. & Begley, D. J. Structure and function of the blood-brain barrier. Neurobiol. Dis. 37(1):13–25 (2010).

45. Li, T., Bourgeois, J.-P., Celli, S., Glacial, F., Le Sourd, A.-M., Mecheri, S., Weksler, B., Romero, I., Couraud, P.-O., Rougeon, F., et al. Cell-penetrating anti-GFAP VHH and corresponding fluorescent fusion protein VHH-GFP spontaneously cross the blood-brain barrier and specifically recognize astrocytes: application to brain imaging. FASEB J. 26(10):3969–79 (2012).

46. Hervé, F., Ghinea, N. & Scherrmann, J.-M. CNS delivery via adsorptive transcytosis. AAPS J. 10(3):455–72 (2008).

47. Abulrob, A., Sprong, H., Van Bergen en Henegouwen, P. & Stanimirovic, D. The blood-brain barrier transmigrating single domain antibody: mechanisms of transport and antigenic epitopes in human brain endothelial cells. J. Neurochem. 95(4):1201–14 (2005).

48. Muruganandam, A., Tanha, J., Narang, S. & Stanimirovic, D. Selection of phage-displayed llama single-domain antibodies that transmigrate across human blood-brain barrier endothelium. FASEB J. 16(2):240–2 (2002).

49. Haqqani, A. S., Delaney, C. E., Tremblay, T.-L., Sodja, C., Sandhu, J. K. & Stanimirovic, D. B. Method for isolation and molecular characterization of extracellular microvesicles released from brain endothelial cells. Fluids Barriers CNS 10(1):4 (2013).

50. Farrington, G. K., Caram-Salas, N., Haqqani, A. S., Brunette, E., Eldredge, J., Pepinsky, B., Antognetti, G., Baumann, E., Ding, W., Garber, E., et al. A novel platform for engineering blood-brain barrier-crossing bispecific biologics. FASEB J. 28(11):4764–4778 (2014).

51. Bjellqvist, B., Hughes, G. J., Pasquali, C., Paquet, N., Ravier, F., Sanchez, J. C., Frutiger, S. & Hochstrasser, D. The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. Electrophoresis 14(10):1023–31 (1993).

52. Bennewitz, M. F. & Saltzman, W. M. Nanotechnology for delivery of drugs to the brain for epilepsy. Neurotherapeutics 6(2):323–36 (2009).

53. Brinker, T., Stopa, E., Morrison, J. & Klinge, P. A new look at cerebrospinal fluid circulation. Fluids Barriers CNS 11:10 (2014).

54. Rip, J., Chen, L., Hartman, R., van den Heuvel, A., Reijerkerk, A., van Kregten, J., van der Boom, B., Appeldoorn, C., de Boer, M., Maussang, D., et al. Glutathione PEGylated liposomes: pharmacokinetics and delivery of cargo across the blood-brain barrier in rats. J. Drug Target. 22(5):460–7 (2014).

55. Lindqvist, A., Rip, J., Gaillard, P. J., Björkman, S. & Hammarlund-Udenaes, M. Enhanced brain delivery of the opioid peptide damgo in glutathione pegylated liposomes: A microdialysis study. Mol. Pharm. 10(5):1533–1541 (2013).

56. Salem, H. F., Ahmed, S. M., Hassaballah, A. E. & Omar, M. M. Targeting brain cells with glutathione-modulated nanoliposomes: in vitro and in vivo study. Drug Des. Devel. Ther. 9:3705–27 (2015).

57. Rotman, M., Snoeks, T. J. A. & van der Weerd, L. Pre-clinical optical imaging and MRI for drug development in Alzheimer's disease. Drug Discovery Today: Technologies 8(2–4):e117–e125 (2011).

58. Sand, K. M. K., Bern, M., Nilsen, J., Noordzij, H. T., Sandlie, I. & Andersen, J. T. Unraveling the Interaction between FcRn and Albumin: Opportunities for Design of Albumin-Based Therapeutics. Front. Immunol. 5:682 (2014).

59. Schlachetzki, F., Zhu, C. & Pardridge, W. M. Expression of the neonatal Fc receptor (FcRn) at the blood-brain barrier. J. Neurochem. 81(1):203–6 (2002).

60. Cooper, P. R., Ciambrone, G. J., Kliwinski, C. M., Maze, E., Johnson, L., Li, Q., Feng, Y. & Hornby, P. J. Efflux of monoclonal antibodies from rat brain by neonatal Fc receptor, FcRn. Brain Res. 1534:13–21 (2013).

61. Bainbridge, J. W. B., Smith, A. J., Barker, S. S., Robbie, S., Henderson, R., Balaggan, K., Viswanathan, A., Holder, G. E., Stockman, A., Tyler, N., et al. Effect of gene therapy on visual function in Leber's congenital amaurosis. N. Engl. J. Med. 358(21):2231–9 (2008).

62. Levine, B. L., Humeau, L. M., Boyer, J., MacGregor, R.-R., Rebello, T., Lu, X., Binder, G. K., Slepushkin, V., Lemiale, F., Mascola, J. R., et al. Gene transfer in humans using a conditionally replicating lentiviral vector. Proc. Natl. Acad. Sci. U. S. A. 103(46):17372–7 (2006).

63. LeWitt, P. A., Rezai, A. R., Leehey, M. A., Ojemann, S. G., Flaherty, A. W., Eskandar, E. N., Kostyk, S. K., Thomas, K., Sarkar, A., Siddiqui, M. S., et al. AAV2-GAD gene therapy for advanced Parkinson's disease: a double-blind, sham-surgery controlled, randomised trial. Lancet. Neurol. 10(4):309–19 (2011).

64. Allen, P. J. & Feigin, A. Gene-based therapies in Parkinson's disease. Neurotherapeutics 11(1):60–7 (2014).

65. Lee, Y. K., Brewer, J. W., Hellman, R. & Hendershot, L. M. BiP and immunoglobulin light chain cooperate to control the folding of heavy chain and ensure the fidelity of immunoglobulin assembly. Mol. Biol. Cell 10(7):2209–19 (1999).

66. Wahner-Roedler, D. L. & Kyle, R. A. Heavy chain diseases. Best Pract. Res. Clin. Haematol. 18(4):729–746 (2005).

67. Muyldermans, S., Atarhouch, T., Saldanha, J., Barbosa, J. A. & Hamers, R. Sequence and structure of VH domain from naturally occurring camel heavy chain immunoglobulins lacking light chains. Protein Eng. 7(9):1129–35 (1994).

68. Nguyen, V. K., Hamers, R., Wyns, L. & Muyldermans, S. Loss of splice consensus signal is responsible for the removal of the entire CH1 domain of the functional camel IGG2A heavy-chain antibodies. Mol. Immunol. 36(8):515–524 (1999).

69. Robberson, B. L., Cote, G. J. & Berget, S. M. Exon definition may facilitate splice site selection in RNAs with multiple exons. Mol. Cell. Biol. 10(1):84–94 (1990).

70. Comi, G. P., Ciafaloni, E., de Silva, H. A., Prelle, A., Bardoni, A., Rigoletto, C., Robotti, M., Bresolin, N., Moggio, M. & Fortunato, F. A G+1toA transversion at the 5' splice site of intron 69 of the dystrophin gene causing the absence of peripheral nerve Dp116 and severe clinical involvement in a DMD patient. Hum. Mol. Genet. 4(11):2171–4 (1995).

71. Nguyen, V. K., Zou, X., Lauwereys, M., Brys, L., Brüggemann, M. & Muyldermans, S. Heavy-chain only antibodies derived from dromedary are secreted and displayed by mouse B cells. Immunology 109(1):93–101 (2003).

72. Muyldermans, S., Cambillau, C. & Wyns, L. Recognition of antigens by single-domain antibody fragments: the superfluous luxury of paired domains. Trends Biochem. Sci. 26(4):230–235 (2001).

73. Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Songa, E. B., Bendahman, N. & Hamers, R. Naturally occurring antibodies devoid of light chains. Nature 363(6428):446–448 (1993).

74. Feige, M. J., Gräwert, M. A., Marcinowski, M., Hennig, J., Behnke, J., Ausländer, D., Herold, E. M., Peschek, J., Castro, C. D., Flajnik, M., et al. The structural analysis of shark IgNAR antibodies reveals evolutionary principles of immunoglobulins. Proc. Natl. Acad. Sci. U. S. A. 111(22):8155–60 (2014).

75. Flajnik, M. F. & Dooley, H. The generation and selection of single-domain, v region libraries from nurse sharks. Methods Mol. Biol. 562:71–82 (2009).

76. Flajnik, M. F., Deschacht, N. & Muyldermans, S. A case of convergence: why did a simple alternative to canonical antibodies arise in sharks and camels? PLoS Biol. 9(8):e1001120 (2011).

77. Smolarek, D., Bertrand, O. & Czerwinski, M. Variable fragments of heavy chain antibodies (VHHs): A new magic bullet molecule of medicine? Postepy Higieny i Medycyny Doswiadczalnej 66:348–358 (2012).

78. Kovaleva, M., Ferguson, L., Steven, J., Porter, A. & Barelle, C. Shark variable new antigen receptor biologics - a novel technology platform for therapeutic drug development. Expert Opin. Biol. Ther. 14(10):1527–39 (2014).

79. de Marco, A. Biotechnological applications of recombinant single-domain antibody fragments. Microb. Cell Fact. 10:44 (2011).

80. Gustafsen, C., Glerup, S., Pallesen, L. T., Olsen, D., Andersen, O. M., Nykjær, A., Madsen, P. & Petersen, C. M. Sortilin and SorLA display distinct roles in processing and trafficking of amyloid precursor protein. J. Neurosci. 33(1):64–71 (2013).

81. Andersen, O. M., Reiche, J., Schmidt, V., Gotthardt, M., Spoelgen, R., Behlke, J., von Arnim, C. a F., Breiderhoff, T., Jansen, P., Wu, X., et al. Neuronal sorting protein-related receptor sorLA/LR11 regulates processing of the amyloid precursor protein. Proc. Natl. Acad. Sci. U. S. A. 102(38):13461–13466 (2005).

82. Schmidt, V., Sporbert, A., Rohe, M., Reimer, T., Rehm, A., Andersen, O. M. & Willnow, T. E. SorLA/LR11 Regulates Processing of Amyloid Precursor Protein via Interaction with Adaptors GGA and PACS-1. J. Biol. Chem. 282(45):32956–32964 (2007).

83. Rohe, M., Hartl, D., Fjorback, A. N., Klose, J. & Willnow, T. E. SORLA-mediated trafficking of TrkB enhances the response of neurons to BDNF. PLoS One 8(8):e72164 (2013).

84. Rohe, M., Carlo, A.-S., Breyhan, H., Sporbert, A., Militz, D., Schmidt, V., Wozny, C., Harmeier, A., Erdmann, B., Bales, K. R., et al. Sortilin-related receptor with A-type repeats (SORLA) affects the amyloid precursor protein-dependent stimulation of ERK signaling and adult neurogenesis. J. Biol. Chem. 283(21):14826–34 (2008).

85. Dodson, S. E., Andersen, O. M., Karmali, V., Fritz, J. J., Cheng, D., Peng, J., Levey, A. I., Willnow, T. E. & Lah, J. J. Loss of LR11/SORLA enhances early pathology in a mouse model of amyloidosis: evidence for a proximal role in Alzheimer's disease. J. Neurosci. 28(48):12877–86 (2008).

86. Mehmedbasic, A., Christensen, S. K., Nilsson, J., Rüetschi, U., Gustafsen, C., Poulsen, A. S. A., Rasmussen, R. W., Fjorback, A. N., Larson, G. & Andersen, O. M. SorLA Complement-type Repeat Domains Protect the Amyloid Precursor Protein against Processing. J. Biol. Chem. 290(6):3359–3376 (2015).

87. Chasseigneaux, S. & Allinquant, B. Functions of Aβ, sAPPα and sAPPβ : similarities and differences. J. Neurochem. 120 Suppl:99–108 (2012).

88. Villemagne, V. L., Klunk, W. E., Mathis, C. a, Rowe, C. C., Brooks, D. J., Hyman, B. T., Ikonomovic, M. D., Ishii, K., Jack, C. R., Jagust, W. J., et al. Aβ Imaging: feasible, pertinent, and vital to progress in Alzheimer's disease. Eur. J. Nucl. Med. Mol. Imaging 39(2):209–19 (2012).

89. Klunk, W. E., Engler, H., Nordberg, A., Wang, Y., Blomqvist, G., Holt, D. P., Bergström, M., Savitcheva, I., Huang, G. F., Estrada, S., et al. Imaging Brain Amyloid in Alzheimer's Disease with Pittsburgh Compound-B. Ann. Neurol. 55(3):306–319 (2004).

90. KleinJan, G. H., Bunschoten, A., van den Berg, N. S., Olmos, R. A. V., Klop, W. M. C., Horenblas, S., van der Poel, H. G., Wester, H.-J. & van Leeuwen, F. W. B. Fluorescence guided surgery and tracer-dose, fact or fiction? Eur. J. Nucl. Med. Mol. Imaging 43(10):1857–1867 (2016).

91. Jiang, W., Cossey, S., Rosenberg, J. N., Oyler, G. A., Olson, B. J. S. C. & Weeks, D. P. A rapid live-cell ELISA for characterizing antibodies against cell surface antigens of Chlamydomonas reinhardtii and its use in isolating algae from natural environments with related cell wall components. BMC Plant Biol. 14:244 (2014).

92. Fatima, A., Wang, H., Kang, K., Xia, L., Wang, Y., Ye, W., Wang, J. & Wang, X. Development of VHH antibodies against dengue virus type 2 NS1 and comparison with monoclonal antibodies for use in immunological diagnosis. PLoS One 9(4):e95263 (2014).

93. Kol, S., Kallehauge, T. B., Adema, S. & Hermans, P. Development of a VHH-Based Erythropoietin Quantification Assay. Mol. Biotechnol. 57(8):692–700 (2015).