AAV mediated delivery of a novel anti-BACE1 VHH reduces Abeta in an Alzheimer's disease mouse model

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ABSTRACT

Single domain antibodies (VHH) are potentially disruptive therapeutics, with important biological value for treatment of several diseases, including neurological disorders. However, VHH have not been widely used in the central nervous system (CNS), as it is hard to reach therapeutic levels, both because of their restricted blood-brain-barrier penetration and their apparent rapid clearance from the parenchyma. Here, we propose a gene transfer strategy based on adeno-associated virus (AAV)-based vectors to deliver VHH directly into the CNS, ensuring continuous production at therapeutic levels. As a proof-of-concept, we explored the potential of AAV-delivered VHH to inhibit BACE1, a well-characterized target in Alzheimer's disease. First, we generated a panel of VHHs targeting BACE1. One of them, VHH-B9, showed high selectivity for BACE1 and efficacy in lowering BACE1 activity in vitro. We then went on to demonstrate significant reductions in amyloid beta (Aβ) levels after AAV-based delivery of VHH-B9 into the CNS of a mouse model of cerebral amyloidosis. These results constitute a novel therapeutic approach for neurodegenerative diseases, which is applicable to a range of CNS disease targets.

INTRODUCTION

The high selectivity of monoclonal antibodies (mAbs) offers unique opportunities to target key proteins involved in the etiology of neurodegenerative conditions, such as Parkinson's disease and Alzheimer's disease (AD) (Zhou *et al*, 2011; Panza *et al*, 2014). However, their potential as central nervous system (CNS) therapeutics is largely limited by their inability to cross the blood brain barrier (BBB) (Zafir-Lavie *et al*, 2018), comparatively poor biodistribution through the parenchyma (Freskgård & Urich, 2017), and short half-life (Wang *et al*, 2018). In addition, there is the potential for Fc receptor-mediated immunogenicity, mediated by microglia, which can cause vasogenic edema and cerebral microhemorrhage (Panza *et al*, 2014).

Single variable domain antibodies (VHHs) are increasingly seen as an alternative to mAbs for therapeutic use (Steeland *et al*, 2016; Bannas *et al*, 2017; Gomes *et al*, 2018). In fact, the VHH-based therapeutic Cablivi® (caplacizumab-yhdp), was recently approved for market by the US Food and Drug Administration (Scully et al, 2019) for peripheral treatment of adults with acquired Thrombotic Thrombocytopenic Purpura (aTTP)

One key reason for their attractiveness over conventional mAbs is their small antigen binding site, which allows them to access unique epitopes not available to conventional mAbs, such as enzyme active sites, which are often key drug targets (Hassanzadeh-Ghassabeh *et al*, 2013). In addition, VHH have a much lower immunogenic profile than traditional mAbs, largely due to the lack of an Fc-region.

Although there have been reports that VHH can pass through the BBB (Li *et al*, 2016), the degree of penetration is variable and linked to the intrinsic charge on the protein (Bélanger *et al*, 2019). Unfortunately, the amount reaching the CNS, following peripheral injection, is further compromised by their high rate of peripheral clearance, due to renal excretion (Gainkam *et al*, 2008; Bannas *et al*, 2015). Although VHHs which do actually reach the CNS (or are directly injected into the CNS) typically have a longer half-life than in plasma, detectable levels are still reduced by up to 50% twenty-four hours post-injection (Dorresteijn *et al*, 2015). Genetic delivery of therapeutic VHHs, directly to the CNS, offers a potential solution to these issues, allowing long-term local production (Zafir-Lavie *et al*, 2018).

Adeno-associated virus (AAV)-based vectors are becoming the vehicle of choice for gene therapy applications, due to their high efficiency of gene transfer and excellent safety profile (reviewed extensively in Hudry & Vandenberghe, 2019). The major drawback of AAV vectors is the limited cargo capacity: transgenes larger than 5kb in size are not efficiently packed into the vector (Trapani *et al*, 2014). Therefore, VHH are ideal candidates for AAV-mediated delivery, as they are comparatively small (typically 350 bp in size) and can be easily incorporated into AAV vectors without extensive modifications that can adversely affect their binding properties (Pain *et al*, 2015), which is a common issue with AAV-mediated delivery of mAbs (Wu *et al*, 2010). In theory, it also allows for additional engineering, for example, directing the VHH into specific trafficking pathways to improve target engagement (Dmitriev *et al*, 2016), or incorporating specific proteolysis-promoting sequences to stimulate intracellular degradation of toxic species (Baudisch *et al*, 2018).

In this work, we describe an efficient AAV-based system for delivery of therapeutic VHH into the CNS. As a proof-of-concept strategy, we targeted the β-site amyloid precursor protein-cleaving enzyme 1 (BACE1), which is a key component in amyloid beta peptide (Aβ) production in AD (Vassar *et al*, 1999; Cai *et al*, 2001). In a two-stage process, we first generated a novel VHH, named B9, which effectively inhibits BACE1 *in vitro*. In the second phase, we then used an AAV vector to deliver B9 into the CNS of an AD mouse model, producing a long-term reduction in Aβ production after a single injection. Together, our results demonstrate that VHH against key CNS disease targets can be produced and delivered efficiently using gene therapy vectors. This approach constitutes a unique therapeutic avenue, not only for AD but for the broad spectrum of CNS diseases.

RESULTS

Production and identification of anti-BACE1 VHH

To generate VHH, a dromedary and a llama were immunized with purified human BACE1 ectodomain (amino acids 46-460) (Figure 1A). In total, 16 different clones were identified that bind to BACE1.

Identification of VHH inhibiting BACE1

We screened for a possible effect of VHH on enzyme activity employing an *in vitro* APP cleavage assay (Zhou *et al*, 2011). Sixteen VHHs were recombinantly expressed in bacteria, purified and added to the assay at a final concentration of 5 μM. VHH inhibited BACE1 to varying degrees, with three VHH consistently giving the highest level of inhibition, with half-maximal effective concentrations (EC_{50}) in the nanomolar range: 99.2 nM (VHH-B9), 112.5 nM (VHH-10C4) and 788.7 nM (VHH-4A2) (Figure 1B, C; Appendix Figure S1).

Characterization of VHH-BACE1 binding

More detailed examination of VHH-BACE1 binding was obtained using the surface plasmon resonance (SPR) technique. The equilibrium dissociation constant (K_D) was determined for VHH-B9, VHH-10C4 and VHH-4A2 at both pH 7.0 (representing the pH of the extracellular environment) and pH 4.5 (pH of the endosomal compartment). In both cases, VHH-B9 showed the highest affinity to BACE1 (Figure 1D). Interestingly, binding affinity seemed to be slightly stronger at pH 4.5, which is beneficial, as the majority of APP cleavage is reported to occur in the endosomal system (Sannerud *et al*, 2011; Ben Halima *et al*, 2016). Despite the high binding affinity of VHH-B9 towards human and mouse BACE1, it did not show any binding to mouse BACE2 under the same measurement conditions (Figure 1E), indicating a high degree of selectivity. Based on these results, VHH-B9 was chosen as the principal candidate for further characterization and testing.

We also explored the epitope on BACE1 mediating VHH-B9 binding by performing competition assays with the well-characterized monoclonal antibody 1A11 (Zhou *et al*, 2011). 1A11 was able to compete for BACE1 binding with VHH-B9 suggesting that these two antibodies bind to overlapping epitopes (Appendix Figure S2). The identity of the epitope was established by observing the binding of VHH-B9 to a series of BACE1 mutants in a dot blot assay. Our results suggest that VHH-B9 binds to BACE1 at Helix A and Loop F, which are close to the active site of the enzyme (Appendix Figure S3). Thus, VHH-B9 may act by blocking effective substrate entry

VHH-B9 effectively inhibits neuronal BACE1 in primary neuronal cultures

To test if VHH-B9 inhibits the enzyme in its native membrane environment, we turned to primary neuronal cultures. Cells were transduced by Semliki Forest Virus (SFV) expressing wild type human APP_{695} and then treated for 12 hours with recombinant VHH-B9 at a final concentration of 3 µM. Treatment with VHH-B9 decreased the levels of $sAPP_{\beta}$, CTF_{β}, $A\beta_{1-40}$ and $A\beta_{1-42}$ detected. The levels of full-length APP, however, remained unchanged (Figure 2A, B, C). VHH-B9 inhibits BACE1 in a dose dependent manner (Figure 2D), with an EC_{50} (95% confidence interval) of 85.4 nM (52.5 nM to 125.6 nM) (Figure 2E). Together, these results indicate that VHH-B9 inhibits BACE1 activity in a native neuronal environment. Note that VHH-10C4 and VHH-4A2 were also tested in neuronal cultures. While both these VHHs inhibited BACE1 activity, VHH-B9 was the most effective, as predicted from the *in vitro* assays (Figure 1).

We next investigated the relative levels of BACE1-mediated cleavage of known substrates following treatment with VHH-B9 (Figure 2F, G). For these experiments, the cDNA for VHH-B9 was modified to contain an N-terminal BACE1 signal peptide and a C-terminal cMyc tag. This cDNA sequence was then cloned into a single-stranded AAV expression cassette and packaged into an AAV capsid (AAV-VHH-B9) (Fripont *et al*, 2019), which was used to transduce primary neuronal cultures. The non-selective BACE1 and BACE2 inhibitor Compound J (CpJ) (Esterházy *et al*, 2011) was used as a positive control. Blocking of sAPPβ shedding was observed in neurons upon transduction with AAV-VHH-B9 or CpJ treatment. In contrast, shedding of seizure protein 6 (SEZ6), a well-known neuronal substrate of BACE1

(Pigoni *et al*, 2016), was only inhibited by CpJ and remained unaffected by VHH-B9 (Figure 1F). Next, using primary glial cultures we evaluated the effects of VHH-B9 on BACE2 by assessing the cleavage of two known substrates, Delta and Notch-like epidermal growth factor-related receptor (DNER) and Vascular cell adhesion molecule 1 (VCAM1) (Voytyuk *et al*, 2018b). As expected from our previous binding data (Figure 1E), the shedding of both DNER and VCAM1 was unaffected after transduction with AAV-VHH-B9, whereas CpJ efficiently blocked the shedding of both substrates (Figure 2G).

Viral vector mediated delivery of VHH-B9 reduced Aβ levels in a mouse model of amyloidosis. We next attempted to reduce Aβ production *in vivo* using AAV-based delivery of VHH-B9. In these experiments, an AAV vector encoding GFP was produced for use as a control. AAV vectors were tested in the APPDutch mouse model of cerebral amyloidosis. This is a transgenic line with neuronal overexpression of human E693Q APP, which causes hereditary cerebral hemorrhage with Dutch type amyloidosis. APPDutch mice present an increased $A\beta_{1\text{-}40/42}$ ratio from an early age (Herzig *et al.*, 2004). Hence, we evaluated the activity of VHH-B9 by direct delivery of AAV-VHH-B9 (n=10) or AAV-GFP (control, n=11) vectors in six-weeks-old mice using a bilateral injection of 2 x 10^{10} vector genomes (vg) per injection site (Appendix Figure S4). Three weeks post-delivery, mice were euthanized. In animals showing VHH-B9 expression, colocalization of VHH-B9 with BACE1 was observed (Figure 3A-D), suggesting significant target engagement. ELISA measurements showed that VHH-B9 expression led to a significant decrease in levels of both $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ (Figure 3E, F), indicative of BACE1 inhibition. No apparent toxicity, derived from either the VHH or the vector, was observed, either on animal survival rates, or at the histological level post-mortem (data not shown).

DISCUSSION

We exploited the known strengths of the VHH platform to successfully demonstrate targeting of BACE1, an important CNS target central to AD pathology (Vassar *et al*, 1999; Barão *et al*, 2016; Voytyuk *et al*, 2018b). These strengths include the ability to rapidly and easily identify VHHs that specifically bind the intended target with adequate affinity, under physiological conditions (Steeland *et al*, 2016). Furthermore, by exploiting the high intrinsic stability of VHHs and ease of engineering (Vincke *et al*, 2009), we were able to incorporate a signal peptide into the VHH sequence, allowing intracellular expression and directed trafficking to the endosome, where the majority of BACE1-mediated APP cleavage is thought to occur (Sannerud *et al*, 2011; Ben Halima *et al*, 2016). This was done with the aim of promoting VHH-BACE1 interactions early in the secretory pathway, which may be beneficial as some mutations in APP, such as the *Swedish* mutation (*Swe*), are known to promote processing of APP in the secretory pathway itself (Sasaguri *et al*, 2017). Finally, the small size of VHH allowed easy incorporation into an AAV-based vector (Verhelle *et al*, 2017). Use of such a system facilitated intracellular delivery and continuous VHH expression, with direct parenchymal injection circumventing the shielding effect of the BBB, which has often limited the therapeutic efficiency of antibody therapies for brain disorders (Barão *et al*, 2016). This allowed us to achieve long-term reductions in BACE1 activity and Aβ production in a mouse model of Alzheimer's type amyloidosis.

As BACE1 contributes to 80% of $A\beta_{1\text{-}40}$ production in the brain (Atwal *et al*, 2011). The decreases in steady state Aβ levels that we report here are significant, especially considering they were achieved after a single administration of vector. By way of comparison, a single intracisternal injection of 75 µg VHH-B3a in APPswe/PS1dE9 mice (Dorresteijn *et al*, 2015) showed no significant decrease in the level of soluble $A\beta_{1\text{-}40}$ twenty-four hours post-injection. This presumably reflects the relative high dissociation constant of this VHH for BACE1 (0.3 µM), a gradual decrease in available VHH (possibly as a result of clearance), or a combination of both factors. In contrast, direct injections of conventional mAbs, or systemic administration of mAbs engineered to cross the BBB by receptor-mediated transcytosis, led to BACE1 inhibition in a dose-dependent fashion (Zhou *et al*, 2011; Cheng *et al*, 2013; Ye *et al*, 2017). In these examples, however, effective inhibition was typically limited to a period of up to forty-eight hours post-injection. Taken together, we believe these results largely validate our experimental strategy, although further work will be needed to determine the optimal temporal window for intervention in the disease process.

At present, the levels of Aβ decrease necessary to produce an effect in AD are undetermined (Kennedy *et al*, 2016; Timmers *et al*, 2018; Egan *et al*, 2019) . However, there are reasons to believe that a sustained steady state reduction, similar to that which we report, may be worthwhile pursuing. First, a reduction of 50% in the levels of BACE1 activity is sufficient to significantly reduce Aβ plaques, neuritic burden, and synaptic deficits in mouse models of AD (McConlogue *et al*, 2007), similar to the effects seen with a reduction of 30% in the levels of gamma secretase activity (Li *et al*, 2007). Second, a mutation in human APP, which reduces cleavage by BACE1 (Jonsson *et al*, 2012), leads to a reduction in cerebral Aβ of approximately 20%, with carriers showing lifelong protection against AD and cognitive decline (although issues with the mutation affecting aggregation of Aβ peptides cannot be fully excluded) (Maloney *et al*, 2014; Benilova *et al*, 2014).

Sustained protection through low to moderate levels of CNS-localized BACE1-specific inhibition may well have the additional benefit of reducing, or avoiding, toxicity related to complete loss of BACE1 function, such as hypomyelination (Willem *et al*, 2006), aberrant synaptic homeostasis and plasticity (Filser *et al*, 2015), axon guidance abnormalities (Rajapaksha *et al*, 2011; Cao *et al*, 2012; Ou-Yang *et al*, 2018), impairments in spatial and working memory (Henley *et al*, 2019; Knopman, 2019; Egan *et al*, 2019) and retinal pathology (Cai *et al*, 2012). In this respect, it is important to note that the levels of BACE1 inhibition achieved with AAV-mediated delivery of VHH-B9 did not substantially affect cleavage of the alternative BACE1 substrate seizure protein 6 (SEZ6), which is important for maintenance of dendritic spines and synaptic plasticity (Gunnersen *et al*, 2007; Pigoni *et al*, 2016; Zhu *et al*, 2018). This is most simply explained by the relative amounts of APP and SEZ6 available for cleavage in a mass-action model (Wilhelm *et al*, 2014) . Peripheral effects of BACE1 inhibition on muscle spindle assembly, as well as cross-reactivity with BACE2, Cathepsin D and Cathepsin E, which can lead to impairments in glucose homeostasis, hypopigmentation, seizures and blindness (amongst others), are also avoided (Voytyuk *et al*, 2018b). Crucially, no signs of apparent toxicity from the VHH or viral vector (at the doses used) were observed over 4 weeks post-vector delivery, consistent with predictions on tolerability from other studies (LeWitt *et al*, 2011; Saraiva *et al*, 2016). Long-term studies focusing on these issues will, however, be essential to determine the clinical feasibility of this approach.

Additional experiments will also be needed to determine the effective therapeutic dose of AAV-VHH-B9 required and whether any reduction in total Aβ load leads to improvements in cognitive function. However, if required, modifications to the system can easily be made by taking advantage of the relative ease with which VHH and AAV can be engineered. For example, VHH affinities in the low picomolar range can be achieved using standard affinity maturation techniques (Mahajan *et al*, 2018), or via production of bivalent VHH constructs (Beirnaert *et al*, 2017) that bind distinct epitopes on a given target. Expression levels of a given VHH from a vector-based system can be improved by use of a self-complementary genome configuration, containing multiple copies of the VHH coding sequence (Verhelle *et al*, 2017). Use of specific promoter systems, in combination with cell type specific transcriptional enhancers and inducible elements (Hudry & Vandenberghe, 2019), should also allow temporally controllable and graded levels of VHH expression in cell types of interest. As demonstrated, VHH can be modified to add unique functions, such as signal peptide sequences (Vincke *et al*, 2009). This can be further exploited to include, for example, sequences targeting the VHH-antigen complex for degradation (Caussinus *et al*, 2011). The efficacy of such modifications, for example in reducing Aβ levels, remains to be established. Finally, AAV vectors which cross the BBB at high efficiency and achieve widespread CNS transduction are being developed. These AAV vectors show considerable promise for delivery of therapeutics to the CNS following systemic injection, removing the need for invasive direct intraparenchymal injections or cerebrospinal fluid-based injections, whilst facilitating broad transgene delivery (Deverman *et al*, 2016). In reality, it is likely to be a combination of technological developments in the aforementioned areas that finally makes AAV-mediated delivery of antibodies, or antibody fragments, a clinically relevant option for CNS disorders.

As production costs drop, such single use AAV technology is likely to offer a more cost-effective alternative to regular infusions of recombinant antibodies or anti-sense oligonucleotides.

In summary, using the well characterized read out of BACE1inhibition as a *proof-of-concept*, we have shown the viability of combining VHH with viral vector-mediated delivery to target a specific CNS protein, producing local and statistically significant reduction in Aβ levels, after a single injection. Moving forward, we propose that vector-mediated delivery of VHHs can be successfully exploited for the treatment of a variety of CNS conditions with defined targets, such as Parkinson's disease and amyotrophic lateral sclerosis, which are, at present, untreatable.

FIGURE LEGENDS

Figure 1. Generation and *in vitro* characterization of anti-BACE1 VHH.

A. Schematic summarizing the procedure used to generate anti-BACE1 VHHs. A dromedary and a llama were immunized with recombinant human BACE1 ectodomain (amino acids 46-460). Blood lymphocytes from the immunized animals were collected for RNA extraction. cDNA was prepared and the variable fragments of heavy chain only IgGs were amplified by RT-PCR and purified using agarose gel electrophoresis. cDNAs encoding VHH were cloned into the pHEN4 phagemid vector and phage libraries were prepared. Three rounds of consecutive phage panning were performed to enrich phage particles that bound recombinant BACE1 in an ELISA assay. Binding to BACE1 was confirmed in a phage ELISA. Finally, BACE1 binding VHHs were purified and further characterized by the MBP-C125sw enzymatic assay.

B. VHHs inhibiting BACE1 were identified with an *in vitro* assay, which uses the maltose binding protein (MBP) fused to a fragment of human APP containing the *Swedish* mutation (APP amino acids 571-965: K670M/N671L) (MBP-C125APPsw assay). VHHs were recombinantly expressed in bacteria, purified and added to the assay at a final concentration of 5 μM. VHH-B9, VHH-10C4 and VHH-4A2 consistently inhibited BACE1 activity, compared to PBS or control VHH (Aβ3 and BCIILP, raised against Aβ peptide and β-lactamase BCII 659/H, respectively). Values are mean ± range, n=2.

C. Dose-response curves for anti-BACE1 VHHs in the MBP-C125APPsw enzymatic assay. The EC_{50} values (95% confidence interval) for VHH-B9, VHH-10C4 and VHH-4A2 are 99.2 nM (83.8-117.5 nM), 112.5 nM (101.1-125.2 nM) and 788.7 nM (682.7-911.2 nM), respectively. Values are mean ± S.D., n=3.

D. Binding affinities of selected VHHs for BACE1. Measurements were made on a BIAcore instrument, using purified BACE1 ectodomain coupled to a CM5 chip. Binding constants were calculated at both pH 7.0 and pH 4.5 to confirm that the selected VHHs bind BACE1 under physiological conditions (extracellular space or trafficking endosome, respectively).

E. Binding affinities of VHH-B9 for BACE1 and BACE2.

Figure 2. Specific inhibition of BACE1-mediated APP cleavage in primary neuronal cultures using VHH.

A, B, C. Primary cultured neurons were transduced by Semliki Forest virus (SFV) expressing wild type human APP695 and treated with 3 μM of the indicated VHHs. PBS and anti-GFP VHH were used as controls. (A) Western blot analysis of conditioned media for sAPPα and sAPPβ, as well as cell extracts for full length APP, CTFα and CTFβ. (B) ELISA measurement of $A\beta_{1\text{-}40}$ in conditioned media. (C) ELISA measurement of $A\beta_{1\text{-}42}$ in conditioned media. Values are mean ± S.E.M., n=3 cultures for each analysis. One-way ANOVA, *** p<0.0001, **p<0.01, *p <0.5.

D, E. Dose-dependent inhibition of BACE1 in primary cultured neurons by VHH-B9. Cultured neurons were transduced by SFV expressing wild type human APP695 and treated with PBS (control: CT) or decreasing concentrations of VHH-B9, ranging from 10 μM to 0.7 nM. (D) Western blot analysis of conditioned media for sAPPα and sAPPβ, as well as cell extracts for full length APP, CTFα and CTFβ. (E) Conditioned media was analyzed by ELISA to assess levels of $A\beta_{1\text{-}40}$. Values are mean ± S.D., n=3 cultures for each analysis. The EC_{50} value (95 % confidence interval) was estimated as 85.4 nM (52.5 - 125.6 nM).

F. Primary cultured neurons were transduced with an AAV vector driving the expression of VHH-B9. Compound J (CpJ) was used as control. VHH-B9 inhibited APP cleavage, as seen by a decrease in sAPPβ production. However, no decrease in SEZ6 shedding was observed. In contrast CpJ successfully inhibited both APP and SEZ6 shedding. Actin was used as a loading control. Anti-cMyc tag and anti-llama IgG were used for VHH detection. n=3 cultures for each analysis.

G. Primary cultured glia were transduced with an AAV vector driving the expression of VHH-B9, or treated with CpJ. VHH-B9 had no effect on the cleavage of the BACE2 substrates DNER and VCAM1. CpJ effectively blocked shedding of both substrates. n=3 cultures for each analysis.

Figure 3. Long-term BACE1 inhibition in APPDutch mice after AAV-VHH delivery significantly reduces amyloid beta load.

A-D. AAV-VHH-B9 vector was injected into the hippocampus of APPDutch mice (n=3) at a dose of $2x10^{10}$ vg per site. Three weeks post-injection, brains were recovered and processed for immunohistochemistry. Representative images of coronal brain sections are shown for staining against BACE1 (magenta) and cMyc (green). Images show colocalization of VHH-B9 with BACE1 in the pyramidal cell layer (Pyr), as well as in the suprapyramidal blade (SPB) and infrapyramidal blade (IPB) of the mossy fibers. The diffuse staining patterns suggest that

VHH-B9 is engaged with BACE1 in internal structures, such as early endosomes. Scale bar, 25 µm.

E, F. APP Dutch mice were injected in the hippocampus with AAV-VHH-B9 (n=11) or the control vector AAV-GFP (n=10). Whole brains were collected 3 weeks post-injection for analysis. Soluble protein was extracted and used for quantification of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels by ELISA. VHH-B9 expression led to a significant decrease in both $A\beta_{1\text{-}40}$ (27.3%, p=0.0168) and $A\beta_{1\text{-}42}$ (27.5%, p=0.0026) levels.

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AUTHOR CONTRIBUTIONS

BDS and MGH conceived the project. LZ, CV, SM, YW, MD and BDS generated and characterized the anti-BACE1 VHH. MYR generated the AAV used in tissue culture experiments; YL and TEG generated the AAV used for *in vivo* experiments. LZ, CM and IV performed tissue culture experiments. MYR and SID performed experiments with APPDutch mice. MYR, LZ, SID, BDS and MGH analyzed the data. MGH, MYR and LZ wrote the manuscript with input from all authors.

CONFLICT OF INTEREST

Johnson and Johnson provided antibodies used in this study. Eli Lilly provided reagents used in the peptide cleavage experiments. However, neither company played a role in the design and execution of the study, or the interpretation of results. BDS has acted as a consultant for Janssen Pharmaceutica and Remynd NV. The remaining authors declare no conflict of interest.

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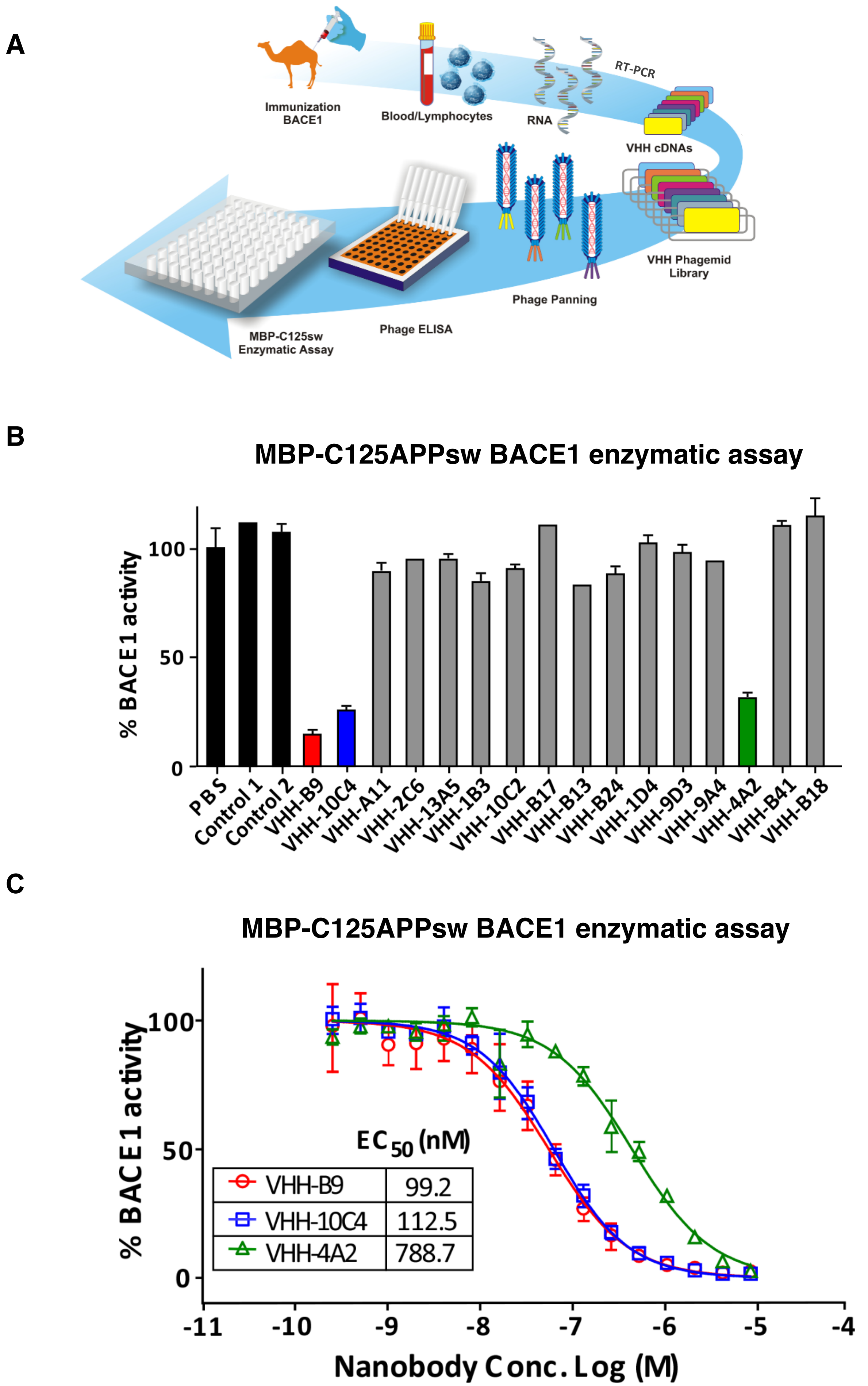
Figure 1. Rincon MY., Zhou L., *et al.*



D

Binding affinities of selected VHH to human BACE1

VHH	pH	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM)
VHH-B9	7.0	2.67×10^5	9.80×10^{-4}	3.7
	4.5	6.62×10^5	1.30×10^{-4}	1.9
VHH-10C4	7.0	1.06×10^5	7.92×10^{-3}	74.7
	4.5	4.51×10^5	1.25×10^{-2}	27.7
VHH-4A2	7.0	4.79×10^5	2.31×10^{-2}	48.2
	4.5	3.97×10^5	8.41×10^{-3}	21.2

E

Binding affinities of VHH-B9 to BACE1 *vs.* BACE2

	Conc. (nM)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM)
mBACE1*	100	2.24×10^5	7.20×10^{-4}	3.2
hBACE1*	100	1.53×10^5	4.43×10^{-4}	2.9
mBACE2	100	NR	NR	NR*

*h, human; m, mouse; NR, no response

Figure 2. Rincon MY., Zhou L., *et al.*

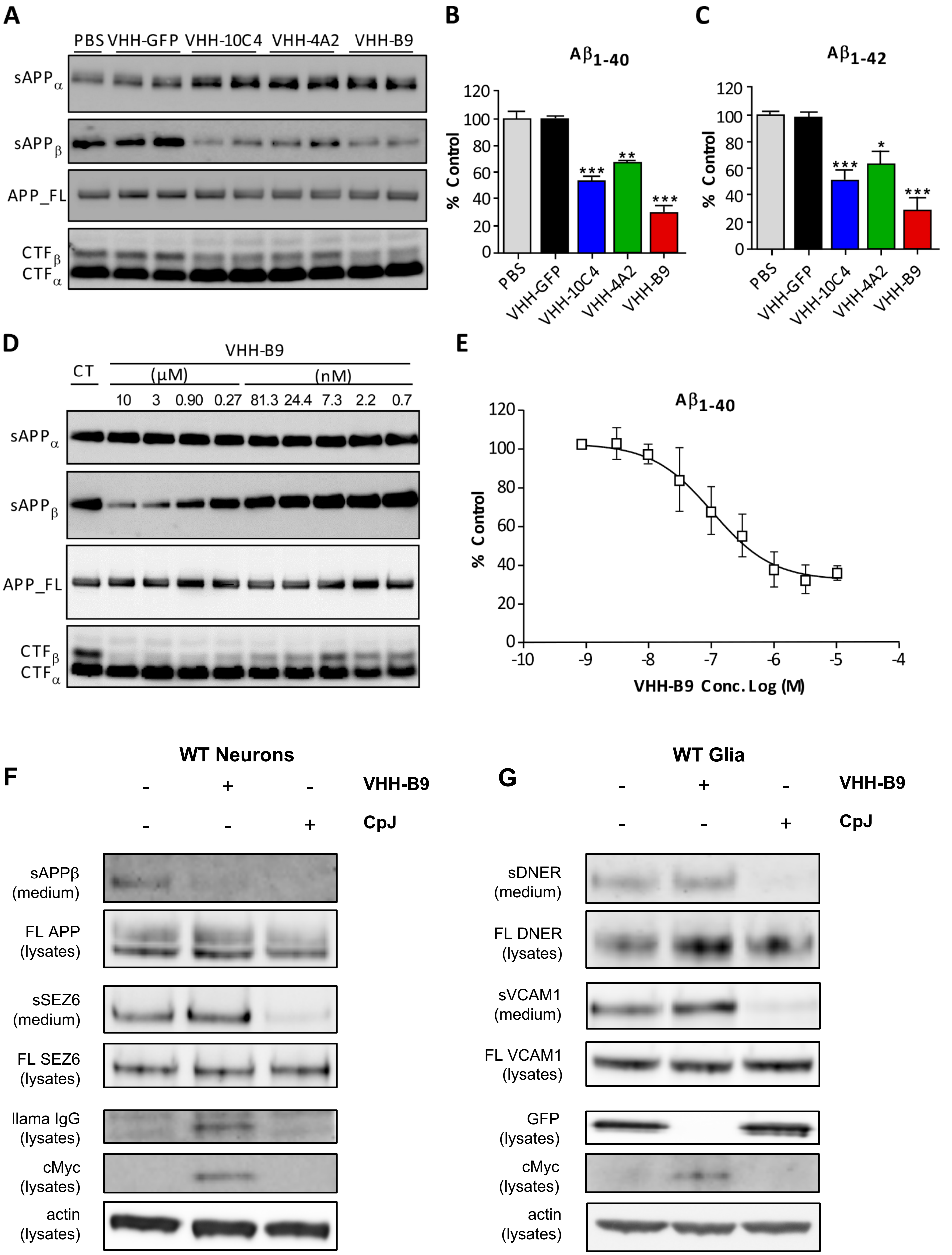


Figure 3. Rincon MY., Zhou L., *et al.*