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Camelid single-domain antibodies: A versatile tool for *in vivo* imaging of extracellular and intracellular brain targets

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ABSTRACT

Detection of intracerebral targets with imaging probes is challenging due to the non-permissive nature of blood-brain barrier (BBB). The present work describes two novel single-domain antibodies (VHHs or nanobodies) that specifically recognize extracellular amyloid deposits and intracellular tau neurofibrillary tangles, the two core lesions of Alzheimer's disease (AD). Following intravenous administration in transgenic mouse models of AD, *in vivo* real-time two-photon microscopy showed gradual extravasation of the VHHs across the BBB, diffusion in the parenchyma and labeling of amyloid deposits and neurofibrillary tangles. Our results demonstrate that VHHs can be used as specific BBB-permeable probes for both extracellular and intracellular brain targets and suggest new avenues for therapeutic and diagnostic applications in neurology.

1. Introduction

Brain penetration of targeted compounds, such as high affinity antibodies, is a prerequisite for the treatment and diagnosis of central nervous system pathologies. However, the entry into the central nervous system of most of blood-circulating molecules is seriously limited by the blood-brain barrier (BBB). It has been shown that only 0.02%–0.1% of conventional immunoglobulins present in serum can penetrate into brain parenchyma [1,2]. Hijacking the BBB receptor-mediated transcytosis system has been envisioned as a way of promoting passage of agents in the brain. Antibodies directed against the transferrin receptor [3], or the insulin receptor [4] have hence been used as Trojan horses to transport therapeutic molecules across the BBB. Unfortunately, receptor-mediated transcytosis targets are highly and broadly expressed in tissues and are often implicated in critical cellular functions, therefore raising possible safety risks [5,6]. Artificial BBB opening by ultrasound [7,8] or by means of BBB permeability enhancers (*e.g.* mannitol [9]) is also an option to facilitate the penetration of compounds within the brain but these methods also have safety limitations [10].

Another approach of targeting intracerebral epitopes relies on the use of homodimeric heavy chain-only antibodies naturally produced in camelidae [11]. These "non-conventional" single-domain antibodies are devoid of light chains and their heavy chain variable domain (VHH) acts as a fully functional binding moiety [12]. VHHs have several unique features such as relatively low molecular weight, high

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production yield, high stability and solubility with the ability to specifically bind target epitopes at subnanomolar affinity. Due to their small size (15 kDa), VHHs occupy only 1/10 of the volume filled by conventional antibodies [11]. Consequently, they can diffuse more rapidly and deeply in fixed tissues [13] and have a better biodistribution profile *in vivo* than conventional antibody fragments [14,15]. Depending on their binding properties, VHHs can be used either to visualize and/or to interact with intracellular targets [16–22].

We recently demonstrated that some VHHs with a basic isoelectric point (pI) are able to readily transmigrate across the BBB *in vivo* after peripheral injection, without the need of any invasive or hazardous procedures (such as opening of the barrier using chemical or ultrasound sonication) [18]. These VHHs can be used as specific transporters as demonstrated by *in vivo* labeling of astrocytes with a VHH-fluorophore conjugate targeting the intracellular protein GFAP [18].

In the present work we engineered two new VHHs that were able to detect extracellular or intracellular pathological brain targets, namely the two main neuropathological lesions in Alzheimer's disease (amyloid plaques and neurofibrillary tangles - NFTs). We designed sensitive and specific anti-Aβ and anti-tau VHHs that allowed detection of plaques and NFTs in brain tissue from patients with Alzheimer's disease and from mouse models of the disease. We also showed that plaques and tangles could be visualized *in vivo* in mice following intravenous administration of these probes.

2. Materials and methods

The main experimental procedures are described here. Detailed materials and methods are provided in Supplementary Materials.

2.1. Human AD brain extracts and immunization

A male alpaca was immunized with 1 mg of fibrillar synthetic Aβ 42 peptide (Bachem).

One alpaca was immunized with tangles-enriched AD extracts (Braak stage V and VI) that were obtained from the NeuroCEB brain bank. This bank is associated to a brain donation program run by a consortium of patient associations (including France Alzheimer Association) and declared to the Ministry of Research and Universities, as requested by French Law. An explicit written consent was obtained for the brain donation in accordance with the French Bioethical Laws. Another alpaca was immunized with a synthetic mono-phosphorylated peptide derived from the C-terminus of the tau protein. A detailed description is provided in Supplementary Materials.

2.2. Library construction, phage preparation and phage display

The library construction was described in [23]. Phages were prepared by transformation of *E. coli* host cells with a recombinant phagemid and a helper phage. A large number of phages (10^{13}) and different blocking buffers were used to perform each round of phage-display. A complete description is provided in Supplementary Materials.

2.3. Selection of specific phage-VHHs and binding epitope determination of monoclonal phage-VHH

The libraries of anti-Aβ and anti-pTau VHHs were panned for reactivity with biotinylated Aβ42, Aβ40 peptides and full-length pTau protein respectively. Epitope mapping of Aβ specific VHHs was performed by the ELISA inhibition test. A detailed protocol is provided in Supplementary Materials.

2.4. Expression of VHHs

The coding sequences of the selected VHHs (R3VQ and A2) in the vector pHEN1 were sub-cloned into a bacterial expression vector pET23d (Novagen) containing a 6-Histidine tag using *Nco*I and *Not*I restriction sites. Purified VHHs were isolated from transformed *E. coli* BL21 (DE3) pLysS cells by immobilized-metal affinity chromatography (IMAC). The full method is described in Supplementary Materials.

2.5. Determination of pI by isoelectric focusing (IEF)

The pI of VHHs was determined by isoelectric focusing using IEF 3–10 Gel (Invitrogen). NEPGHE (non equilibrium pH gradient gel electrophoresis) with sample application at the anode was also used because it allows optimal protein analysis in the basic range of the gel including pI 8.5 to 10.5 (SERVA Gel IEF 3–10 instruction manual).

2.6. Preparation of AlexaFluor 488-conjugated VHH and mAb

The cysteine present in the C-terminal region of VHH and the SH group in the hinge region of reference mAbs (4G8 and muRb86) were used to realize the conjugation with the maleimido-AF488 fluorophore (Invitrogen). A detailed description is provided in Supplementary Materials.

2.7. Animal models

PS2APP mice overexpressing hAPP Swedish mutation combined with PS2 N141I mutation [24,25] were used as animal models harboring Aβ-positive lesions. PS2APP mice start to develop overt Aβ deposition in the brain at approximately 6 months of age.

Tg4510 mice with the hMAPT P301L gene mutation [26] were used as NFT-bearing mouse model. NFT start to appear in the cortex by 4 months of age and one month later in the hippocampus. All animal experiments were performed in accordance with the guidelines established by European Union legislation regarding the use and care of laboratory animals. Four PIs of the present study (B.D., Pi.L., M.V. and M.D.) have received official agreements from the French Ministry of Agriculture to carry out research and experiments on animals.

2.8. Stereotaxic injections of VHHs

Stereotaxic injections of each VHH were performed in anesthetized mice. Two microliters of VHH was injected into the frontal cortex at the rate of 0.2 μl/min. Two or 24 h later, brains were collected for histological analysis. A detailed protocol is provided in Supplementary Materials.

2.9. Immunohistochemistry and double immunofluorescence staining

Immunohistochemistry was primarily performed on 4 μm thick paraffin sections. A pre-treatment with formic acid was performed before neutralization of endogenous peroxidase activity. After incubation with primary and secondary antibodies (see Supplementary Materials), standard immuno-peroxidase method was applied with DAB as final chromogen. Immunohistochemistry on free-floating sections, fresh tissues and VHH-exposed tissues was performed with similar procedures. Double immunofluorescence staining was implemented on paraffin sections using standard protocols. A detailed description is provided in Supplementary Materials.

2.10. Estimation of lesion loads by VHHs and reference antibodies

Comparative lesion loads detected by VHHs *vs* reference antibodies were assessed on immunostained sections obtained from PS2APP and Tg4510 mice. The full method is described in Supplementary Materials.