In Vivo Detection of Amyloid-\(\beta\) Deposits Using Heavy Chain Antibody Fragments in a Transgenic Mouse Model for Alzheimer's Disease

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Accessibility
In Vivo Detection of Amyloid-β Deposits Using Heavy Chain Antibody Fragments in a Transgenic Mouse Model for Alzheimer’s Disease

Rob J. A. Nabuurs¹,⁵, Kim S. Rutgers⁴,⁵, Mick M. Welling¹, Athanasios Metaxas³, Maaike E. de Backer¹, Maarten Rotman², Brian J. Bacskai⁶, Mark A. van Buchem¹, Silvère M. van der Maarel², Louise van der Weed¹,²,⁵

Introduction

Besides neurofibrillary tangles, Alzheimer’s disease (AD) is characterized by cerebral deposition of β-amyloid (Aβ) in so-called senile or diffuse plaques [1]. Similar vascular deposits of Aβ associated with cerebral amyloid angiopathy (CAA) lead to loss of vessel wall integrity increasing the risk of brain haemorrhages [2]. Present in 30% of the non-demented population over 60 years of age, CAA co-exists in 90% of the AD patients and forms an important complication in the development of immunotherapeutic strategies [3–5]. Although, the exact role of Aβ regarding the underlying pathogenes remains unsolved, accumulation is believed to start 20–30 years prior to clinical onset [6,7]. Distinctive in vivo detection of the different Aβ deposits therefore renders important knowledge regarding early diagnosis and preventive therapy development.

Abstract

This study investigated the in vivo properties of two heavy chain antibody fragments (V₈H), ni3A and pa2H, to differentially detect vascular or parenchymal amyloid-β deposits characteristic for Alzheimer’s disease and cerebral amyloid angiopathy. Blood clearance and biodistribution including brain uptake were assessed by bolus injection of radiolabeled V₈H in APP/PS1 mice or wildtype littermates. In addition, in vivo specificity for Aβ was examined in more detail with fluorescently labeled V₈H by circumventing the blood-brain barrier via direct application or intracarotid co-injection with mannitol. All V₈H showed rapid renal clearance (10–20 min). Twenty-four hours post-injection ⁹⁹mTc-pa2H resulted in a small yet significant higher cerebral uptake in the APP/PS1 animals. No difference in brain uptake were observed for ⁹⁹mTc-ni3A or DTPA(¹¹¹In)-pa2H, which lacked additional peptide tags to investigate further clinical applicability. In vivo specificity for Aβ was confirmed for both fluorescently labeled V₈H, where pa2H remained readily detectable for 24 hours or more after injection. Furthermore, both V₈H showed affinity for parenchymal and vascular deposits, this in contrast to human tissue, where ni3A specifically targeted only vascular Aβ. Despite a brain uptake that is as yet too low for in vivo imaging, this study provides evidence that V₈H detect Aβ deposits in vivo, with high selectivity and favorable in vivo characteristics, making them promising tools for further development as diagnostic agents for the distinctive detection of different Aβ deposits.


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† These authors contributed equally to this work.

Currently, a gross differentiation can only be made based on the occipital predilection of CAA, while existing PET ligands, like ¹¹C-PiB, target Aβ in its fibrillar amyloid form rather than specific vascular or parenchymal types of Aβ deposits [9].

Previously, we have selected heavy chain antibody fragments with high affinity specific for either CAA or all types of human Aβ deposits [9]. Derived from the Camelid heavy chain antibody repertoire, which completely lack light chains, their single N-terminal domain (V₈H) is fully capable of antigen binding with affinities comparable with those of conventional antibodies [10,11]. Blood-brain barrier (BBB) passage was shown to be favorable in an in vitro assay [12]; therefore, this study assessed the in vivo characteristics of two distinct Aβ targeting V₈H, ni3A and pa2H, for their potential use to differentially detect AD and CAA. First, pharmacological behaviour and biodistribution were examined after administration of radiolabeled V₈H into a transgenic AD/CAA
mouse model. Secondly, fluorescently labeled VHH were administered after the BBB was circumvented to evaluate their ability to specifically bind Aβ deposits in vivo.

Materials and Methods

Production of ni3A and pa2H

VH1H ni3A and pa2H were selected from respectively a non-immune or an immune library created after immunisation with post-mortem brain parenchyma of a patient with Down’s syndrome. VH1H were subcloned and produced as previously reported including a myc- or VSV-tag for detection and a his-tag for purification [9]. Similarly, pa2H free of any additional peptide tags was commercially produced by overexpression in yeast (BAC, Leiden, the Netherlands).

Animal studies

All studies were performed using 12–16 month old transgenic mice or wildtype littermates from a colony set up using the APPswe/PS1dE9 strain (APP/PS1) (JAX), known to accumulate vascular and parenchymal Aβ deposits [13], and have been approved by the institutional Animal Ethics Committee (DEC) at the Leiden University Medical Center, permit number 09132. Besides standard genotyping, after each experiment amyloid pathology was confirmed by standard Thioflavin T staining.

Human material

Human brain tissue was obtained of AD/CAA patients or controls as confirmed by neuropathological examination in agreement with the guidelines of the ethics committee of the LUMC. Patient anonymity was strictly maintained. All tissue samples were handled in a coded fashion, according to Dutch national ethical guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies).

Murine specificity of the selected VH1H

To evaluate appropriate use of the APP/PS1 mouse model, murine cryosections (10 μm) were stained according previous protocols [9,12] with in addition a standard anti-mouse-to-mouse kit (ARK, Dako Cytomation). Final preparations were analyzed with an automated Pannoramic MIDI microscope (3DHistech).

Biodistribution and clearance

Radiolabeling. VH1H were labeled according to two different protocols. First, his-tagged VH1H were labeled directly with technetium-99m (99mTc) using a previously published protocol [14]. Briefly, 20 μl of VH1H in PBS solution (450–500 ng/μl) was added to 8 μl of an aseptic mixture of 950 mg/l Sn(Cl)2.2H2O and 2 g/l Na4P2O7.10H2O (Technescan PYP, Covidien, Petten, the Netherlands) in saline. After addition of 4 μl of 10 mg/ml of KBH4 (crystalline, Sigma Chemical Co, St. Louis, MO) in 0.1 M NaOH, and 100 μl of Na[99mTcO4] solution (approximately 200–700 MBq/ml, Technekow, Covidien, Petten, the Netherlands) the mixture was gently stirred at room temperature for at least 30 min before use. Analysis of the labeling solution, referred to as 99mTc-VH1H, yielded a radiochemical purity of >95% without detectable unreduced or free 99mTcO4 [15].

Secondly, untagged VH1H were chelated for indium-111 (111In) using diethylene triamine penta-acetic acid (DTPA). Untagged pa2H was chelated in a total volume of 1.0 ml with 20-fold molecular excess of p-SCN-Bn-DTPA (Macrocyclics, Dallas, TX) at pH 8.5 in phosphate buffer for 5 hr at 37.5 °C and purified by dialysis using phosphate buffered saline (PBS). 111In chloride (25 μl, 111 MBq/ml, Covidien, the Netherlands) was added to DTPA-pa2H conjugate (0.1 ml) in 0.25 M ammonium acetate buffer (0.8 ml) at pH 5.5 and incubated for 1 hr at room temperature. The reaction was quenched with 50 mM ethylene diamine tetra-acetic acid (EDTA) (50 μl) to chelate residual non-bound 111In and the radiolabeled antibody was then purified using...
Table 1. Biodistribution of $^{99m}$Tc-ni3A in mice.

<table>
<thead>
<tr>
<th>Tissue/organ</th>
<th>Wildtypes</th>
<th>APP/PS1</th>
<th>Wildtypes</th>
<th>APP/PS1</th>
<th>Wildtypes</th>
<th>APP/PS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>t = 3 hr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>blood</td>
<td>1.202 ± 0.379</td>
<td>1.146 ± 0.137</td>
<td>0.778 ± 0.048</td>
<td>0.808 ± 0.115</td>
<td>0.451 ± 0.073</td>
<td>0.363 ± 0.051</td>
</tr>
<tr>
<td>heart</td>
<td>0.525 ± 0.129</td>
<td>0.508 ± 0.109</td>
<td>0.347 ± 0.049</td>
<td>0.337 ± 0.127</td>
<td>0.252 ± 0.041</td>
<td>0.216 ± 0.014</td>
</tr>
<tr>
<td>lungs</td>
<td>0.850 ± 0.184</td>
<td>0.819 ± 0.208</td>
<td>0.659 ± 0.184</td>
<td>0.743 ± 0.301</td>
<td>0.375 ± 0.113</td>
<td>0.291 ± 0.055</td>
</tr>
<tr>
<td>liver</td>
<td>1.078 ± 0.235</td>
<td>1.000 ± 0.293</td>
<td>1.078 ± 0.188</td>
<td>1.223 ± 0.424</td>
<td>0.568 ± 0.149</td>
<td>0.488 ± 0.164</td>
</tr>
<tr>
<td>spleen</td>
<td>0.590 ± 0.257</td>
<td>0.531 ± 0.084</td>
<td>0.792 ± 0.144</td>
<td>0.753 ± 0.291</td>
<td>0.397 ± 0.056</td>
<td>0.465 ± 0.234</td>
</tr>
<tr>
<td>muscle</td>
<td>0.171 ± 0.075</td>
<td>0.120 ± 0.069</td>
<td>0.111 ± 0.088</td>
<td>0.086 ± 0.022</td>
<td>0.043 ± 0.007</td>
<td>0.048 ± 0.008</td>
</tr>
<tr>
<td>cerebrum</td>
<td>0.035 ± 0.009</td>
<td>0.035 ± 0.007</td>
<td>0.031 ± 0.007</td>
<td>0.035 ± 0.008</td>
<td>0.018 ± 0.003</td>
<td>0.019 ± 0.001</td>
</tr>
<tr>
<td>cerebellum</td>
<td>0.073 ± 0.031</td>
<td>0.063 ± 0.009</td>
<td>0.098 ± 0.009</td>
<td>0.096 ± 0.014</td>
<td>0.029 ± 0.005</td>
<td>0.026 ± 0.002</td>
</tr>
<tr>
<td>cerebrum/blood ratio</td>
<td>0.030 ± 0.003</td>
<td>0.030 ± 0.004</td>
<td>0.040 ± 0.010</td>
<td>0.043 ± 0.004</td>
<td>0.040 ± 0.001</td>
<td>0.053 ± 0.008*</td>
</tr>
<tr>
<td>cerebrum/muscle ratio</td>
<td>0.242 ± 0.142</td>
<td>0.335 ± 0.116</td>
<td>0.407 ± 0.270</td>
<td>0.428 ± 0.171</td>
<td>0.422 ± 0.029</td>
<td>0.403 ± 0.100</td>
</tr>
</tbody>
</table>

* = P<0.05 wildtype mice compared to APP/PS1 mice.

A bolus injection of 2 μg $^{99m}$Tc-ni3A was administered intravenously into 12–14 month old APP/PS1 mice or their wild type littermates. At three time points after injection the animals were sacrificed and various tissues and entire organs were removed, weighed and counted for radioactivity. Values are expressed as a percentage of the injected dose per gram tissue (mean ± SD). doi:10.1371/journal.pone.0038284.t001

Table 2. Biodistribution of radiolabeled pa2H in mice.

<table>
<thead>
<tr>
<th>Tissue/organ</th>
<th>Wildtypes</th>
<th>APP/PS1</th>
<th>Wildtypes</th>
<th>APP/PS1</th>
<th>Wildtypes</th>
<th>APP/PS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>t = 3 hr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>blood</td>
<td>0.566 ± 0.003</td>
<td>0.654 ± 0.015</td>
<td>1.009 ± 0.054</td>
<td>1.244 ± 0.123</td>
<td>0.575 ± 0.084</td>
<td>0.696 ± 0.049</td>
</tr>
<tr>
<td>heart</td>
<td>0.273 ± 0.121</td>
<td>0.240 ± 0.017</td>
<td>0.623 ± 0.101</td>
<td>0.763 ± 0.031</td>
<td>0.367 ± 0.059</td>
<td>0.393 ± 0.007</td>
</tr>
<tr>
<td>lungs</td>
<td>0.843 ± 0.256</td>
<td>0.537 ± 0.010</td>
<td>0.930 ± 0.242</td>
<td>1.088 ± 0.035</td>
<td>0.620 ± 0.160</td>
<td>0.622 ± 0.031</td>
</tr>
<tr>
<td>liver</td>
<td>2.615 ± 0.796</td>
<td>1.866 ± 0.016</td>
<td>3.014 ± 0.201</td>
<td>3.392 ± 1.932</td>
<td>1.430 ± 0.402</td>
<td>1.161 ± 0.470</td>
</tr>
<tr>
<td>spleen</td>
<td>1.151 ± 0.503</td>
<td>1.319 ± 0.060</td>
<td>6.498 ± 1.623</td>
<td>6.258 ± 0.208</td>
<td>3.584 ± 1.381</td>
<td>1.747 ± 1.000</td>
</tr>
<tr>
<td>muscle</td>
<td>0.356 ± 0.179</td>
<td>0.054 ± 0.006</td>
<td>0.174 ± 0.022</td>
<td>0.347 ± 0.026</td>
<td>0.102 ± 0.023</td>
<td>0.113 ± 0.018</td>
</tr>
<tr>
<td>cerebrum</td>
<td>0.014 ± 0.003</td>
<td>0.017 ± 0.001</td>
<td>0.033 ± 0.005</td>
<td>0.044 ± 0.004</td>
<td>0.027 ± 0.004</td>
<td>0.038 ± 0.002*</td>
</tr>
<tr>
<td>cerebellum</td>
<td>0.023 ± 0.001</td>
<td>0.026 ± 0.001</td>
<td>0.054 ± 0.016</td>
<td>0.067 ± 0.001</td>
<td>0.030 ± 0.007</td>
<td>0.045 ± 0.000**</td>
</tr>
<tr>
<td>cerebrum/blood ratio</td>
<td>0.025 ± 0.005</td>
<td>0.026 ± 0.003</td>
<td>0.033 ± 0.004</td>
<td>0.035 ± 0.004</td>
<td>0.047 ± 0.003</td>
<td>0.055 ± 0.008</td>
</tr>
<tr>
<td>cerebrum/muscle ratio</td>
<td>0.083 ± 0.081</td>
<td>0.309 ± 0.067</td>
<td>0.190 ± 0.007</td>
<td>0.177 ± 0.135</td>
<td>0.270 ± 0.032</td>
<td>0.346 ± 0.377</td>
</tr>
</tbody>
</table>

* = P<0.05 wildtype mice compared to APP/PS1 mice.

A bolus injection of 2 μg radiolabeled pa2H was administered intravenously into 12–14 month old APP/PS1 mice or their wild type littermates. At three or one time points after injection of radiolabeled pa2H respectively with or without additional peptide tags, the animals were sacrificed and various tissues and entire organs were removed, weighed and counted for radioactivity. Values are expressed as a percentage of the injected dose per gram tissue (mean ± SD). doi:10.1371/journal.pone.0038284.t002
points between 3–90 minutes post-injection of radiolabeled VHH into transgenic or wildtype mice (n = 4–6). Combined with the cardiac blood samples corresponding half-lives were calculated using GraphPad Prism.

Similarly, 10 μl samples obtained at 10 and 90 minutes post-injection were mixed with 90 μl heparin (34 U/ml saline) and 900 μl PBS. Centrifugation for 10 minutes at 7,000 rpm separated plasma from the cell pellet. Radioactivity was measured separately to determine the blood distribution of radiolabeled VHH over time.

Specificity of radiolabeled pa2H. Aβ specificity of pa2H-his after 99mTc-radiolabeling was tested by quantitative competition autoradiography. Human and murine brain cryosections (20 μm) were blocked with 1% bovine serum albumin (BSA)/PBS at 37°C for 1 hour followed by similar application of the labeling solution, which was diluted to 1 μg/ml by 1%BSA/PBS with or without additional 1 hour pre-incubation with excess monomeric or fibrillar Aβ1–40 (rPeptide) at 37°C. Fibrils were produced using existing protocols [16].

After rinsing 3 times with PBS, radioactivity was counted for 15 minutes by a gamma camera (Toshiba GCA7100/UI). A similar region of interest was fitted for each scintigram to assess binding of 99mTc-pa2H-his with 0.1 ml of diluted labeling solution as a reference. Binding was expressed as the % of radioactivity compared to the section without any competitor. Experiments were performed in triplicate.

In vivo Aβ targeting by VHH Fluorescent labeling. Tagged VHH were fluorescently labeled with Alexa Fluor 594 protein labeling kit (Molecular Probes, Invitrogen) according to the manufacturer’s guidelines, except using only half of the recommended amount of dye. Briefly spun to remove possible aggregates, extensive dialysis removed (20 μm) were blocked with 1% bovine serum albumin (BSA)/PBS at 37°C for 1 hour followed by similar application of the labeling solution, which was diluted to 1 μg/ml by 1%BSA/PBS with or without additional 1 hour pre-incubation with excess monomeric or fibrillar Aβ1–40 (rPeptide) at 37°C. Fibrils were produced using existing protocols [16].

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After rinsing 3 times with PBS, radioactivity was counted for 15 minutes by a gamma camera (Toshiba GCA7100/UI). A similar region of interest was fitted for each scintigram to assess binding of 99mTc-pa2H-his with 0.1 ml of diluted labeling solution as a reference. Binding was expressed as the % of radioactivity compared to the section without any competitor. Experiments were performed in triplicate.

### Table 3. Blood half lives of radiolabeled VHH.

<table>
<thead>
<tr>
<th>VHH</th>
<th>genotype</th>
<th>Fast ( t_{1/2} ) (min) (95% C.I)%</th>
<th>Slow ( t_{1/2} ) (min) % (95% C.I)%</th>
</tr>
</thead>
<tbody>
<tr>
<td>99mTc-ni3A</td>
<td>APP/PS1</td>
<td>14.71 (8.65–49.13)</td>
<td>89.7 (10.18–91.80)</td>
</tr>
<tr>
<td>Wildtype</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>99mTc-pa2H</td>
<td>APP/PS1</td>
<td>21.89 (14.24–39.38)</td>
<td>79.8 (20.2–97.50)</td>
</tr>
<tr>
<td>Wildtype</td>
<td>10.78 (7.27–20.76)</td>
<td>87.1</td>
<td>5861 (12.9–969.3)</td>
</tr>
<tr>
<td>DTPA(111In)-pa2H</td>
<td>APP/PS1</td>
<td>19.69 (12.63–44.60)</td>
<td>100 (96.9–100)</td>
</tr>
<tr>
<td>Wildtype</td>
<td>15.83 (9.30–53.37)</td>
<td>100 (96.9–100)</td>
<td></td>
</tr>
</tbody>
</table>

Half lives were determined by fitting a one or a two phase exponential decay model based on blood obtained from both tail vein and cardiac punctate at several time points after intravenous bolus injection of 2 μg radiolabeled VHH in 12–14 month old APP/PS1 mice and wildtype littermates, as depicted in Figure 2. Please note that DTPA(111In)-pa2H was produced without any additional peptide tags.

doi:10.1371/journal.pone.0038284.t003

### Table 4. Blood distribution of 99mTc-pa2H.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time p.i.</th>
<th>Fraction</th>
<th>APP/PS1</th>
<th>Wildtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>99mTc-pa2H</td>
<td>10</td>
<td>Plasma</td>
<td>88.9 (6.2)</td>
<td>80.3 (5.2)</td>
</tr>
<tr>
<td>Cell Pellet</td>
<td>11.1</td>
<td>19.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>Plasma</td>
<td>83.6 (8.7)</td>
<td>72.0 (8.2)</td>
<td></td>
</tr>
<tr>
<td>Cell Pellet</td>
<td>16.4</td>
<td>28.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

At different time point after bolus injection of 99mTc-pa2H blood collected from the tail vein of 12–14 month old APP/PS1 mice or wildtype littermates. Separated into the cell pellet and plasma, samples were counted for radioactivity. Fractions are expressed in percentage of total activity at that time point. No significant differences were calculated using a student t-test \((p<0.05)\).

doi:10.1371/journal.pone.0038284.t004
Table 5. Quantitative autoradiography.

<table>
<thead>
<tr>
<th>Brain tissue</th>
<th>Binding of 99mTc-VHH</th>
<th>Competition binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng (± sd)</td>
<td>ng (± sd)</td>
</tr>
<tr>
<td>APP/PS1</td>
<td>98.8 (±20.7)*</td>
<td>60.1 (±22.2)</td>
</tr>
<tr>
<td>Wildtype</td>
<td>86.4 (±14.8)</td>
<td>56.4 (±19.5)</td>
</tr>
<tr>
<td>AD human</td>
<td>190.1 (±73.5)*</td>
<td>81.4 (±N.D.)</td>
</tr>
<tr>
<td>Control human</td>
<td>102.3 (±30.2)</td>
<td>27.2 (±N.D.)</td>
</tr>
</tbody>
</table>

Differences in radioactivity were measured after application of 1 μg 99mTc-pa2H to human and murine APP/PS1 brain sections.

*Statistical difference (p<0.05) between either murine or human control versus Aβ bearing sections.

doi:10.1371/journal.pone.0038284.t005

excess free label. The labeling degree and protein concentration (200–600 ng/μl) were determined using the Nanodrop ND1000 (Isogen Life Sciences). Protein integrity was confirmed by mass spectrometry.

Immunofluorescence using VHH-Alexa594. To examine whether the fluorescent labeling affected antigen recognition, human and murine cryosections (10 μm) were rinsed with PBS, fixed in ice-cold acetone for 10 minutes before overnight incubation with VHH-Alexa594 in 1% BSA/PBS in a wet chamber. Washed 3×5 minutes with PBS, sections were mounted and analyzed using a fluorescence microscope (Leica DM5500B).

In vivo Aβ imaging by topical application. Four APP/PS1 animals received permanent cranial windows to allow serial in vivo imaging of the brain by multiphoton microscopy. Animals were anaesthetized using 2% isoflurane gas inhalation, and the exposed skull was partly replaced by a round glass coverslip glued into place using Krazyglue® according to previous surgical protocols [17,18]. Prior to fixation of the cranial window, a drop of 40–60 μl of VHH-Alexa594 (275–400 ng/μl) was applied directly onto the exposed brain for 30 minutes and briefly rinsed with PBS. Colocalization with the Aβ deposits was based either upon their typical green autofluorescence or by intraperitoneal injections of Methoxy-X04 one day prior surgery [16]. Animals were imaged immediately following surgery, which was typically less than 90 minutes after beginning of the procedure, and re-imaged under isoflurane anaesthesia (2%) for several days to study the washout. Images were acquired with a Bio-Rad 1024 multiphoton microscope equipped with a Ti:Sapphire laser (Mai Tai, Spectra Physics) and external detectors (Hamamatsu Photonics). Areas were imaged to approximately 200 μm deep in 5 μm steps with a 20× objective (UMPlanFl, NA = 0.95; Olympus). Maximum intensity projections were reconstructed using ImageJ.

Specific in vivo Aβ binding after BBB disruption. A systemic approach to study the in vivo behaviour of the VHH throughout a larger area within the brain involved intracarotid infusion (60 μl/min) of 100 μl pa2H-his-Alexa394 along with 600 μl 15% mannitol selectively into the right carotid artery to disrupt the BBB [19]. At t = 2 and 24 hours post-injection, transgenic (n = 9) and wildtype animals (n = 3) were euthanized (Euthanasol, AST Pharma), and perfused with 4% paraformaldehyde (PFA). Resected brains were stored in 4% PFA with 10% sucrose for 4 hours followed by overnight fixation in 4% PFA with 30% sucrose. Next, the brains were snap frozen and sectioned completely to obtain consecutive 30-μm-thick cryosections. Besides standard Thiouilavin T staining for amyloid, adjacent sections were immunostained for Aβ (6F/3D, DakoCytomation) [20] with 1:100 goat-antimouse-Alexa488 (Invitrogen) to assess colocalization. Images obtained by a Leica DM5500B microscope were merged using Adobe Photoshop CS3.

Results

Murine specificity of the selected VHH

Immunostained brain sections of aged APP/PS1 and wildtype littermates using tagged VHH ni3A and pa2H were made to assess their capacity to selectively recognize different types of deposits. (Figure 1) Pa2H stained positive for all forms of Aβ deposits. In this transgenic mouse model, ni3A did not show selective affinity for vascular Aβ; both vascular and parenchymal Aβ deposits were clearly labeled. Compared to ni3A, equivalent staining protocols with pa2H resulted in higher specificity for Aβ combined with a low unspecific background binding. For neither VHH specific affinity was detected within the brain sections of wildtype animals.

Biodistribution and clearance

Biodistribution and brain uptake. The distribution of a bolus injection of radiolabeled tagged ni3A and pa2H over time is shown in Table 1 and 2. No significant differences in organ uptake between wildtype and transgenic animals were found, except for the brain uptake of 99mTc-pa2H after 24 hours. Although the amount was low (0.038%I.D./g), cerebral uptake was 40% higher in the transgenic animals. The cerebrum/blood ratio did not differ, indicating that this difference was not caused by different VHH concentrations within the blood pool. For the cerebellum similar results were found. Repeated experiments for this particular endpoint resulted in similar findings.

To investigate whether these findings were not confounded by either the non-specific radiolabeling procedure or the presence of additional peptide tags, the biodistribution experiment was repeated with untagged DTPA[111In]-pa2H. (Table 2) With this labeling protocol, we no longer observed a significantly higher cerebral uptake in amyloid-bearing mice. Regardless of the tag, the majority of radiolabeled VHH was excreted via the kidneys. Cellular involvement as shown by distinctive hepatic clearance or splenic activity was low. In comparison to 99mTc-ni3A, 99mTc-pa2H showed about 3 times higher clearance via liver and spleen. Also, the clearance rate for 99mTc-pa2H was lower, independent of genotype. However, within the first 3 hours 99mTc-ni3A resulted in a higher general organ uptake, with exception of the aforementioned liver and spleen.

Blood clearance and analysis. Blood clearance of the tagged 99mTc-VHH consisted of a fast and a slow component. (Figure 2A) In general, the majority of the radiolabeled VHH was cleared from the blood with a half-life of 10–20 minutes (Table 3). The actual half-life of the slow component of 99mTc-VHH could only be calculated with limited accuracy, since the half-life was longer than the blood sampling period. In line with the above biodistribution, six hours post-injection, the blood levels of 99mTc-pa2H were remarkably higher compared to earlier time points, which is characteristic for a second passage. Within the first 90 minutes about 80% of the 99mTc-VHH remained within the blood plasma, indicating that no significant cellular uptake occurred. (Table 4)

In contrast to tagged 99mTc-VHH, the blood clearance of untagged DTPA[111In]-pa2H was mono-exponential, with a similar rapid clearance within 20 minutes, but without a slow component. (Figure 2B)
Specificity of 99mTc-pa2H. After radiolabeling of the tagged pa2H, its specificity for Ab was unaffected, as shown by scintigraphic analysis; binding of $^{99}$Tc-pa2H was higher in those sections including Ab. (Table 5) Furthermore, binding was significantly (p < 0.001) reduced when the tracer was pre-incubated with either monomeric or fibrillar Ab.

In vivo Ab targeting by Vh4

In vivo Ab imaging by topical application. After direct application onto the exposed mouse brain, fluorescent Vh4H were followed up for at least 48 hours by in vivo multiphoton microscopy. (Figure 3) Specific in vivo labeling of Ab plaques by ni3A-Alexa594 was initially confirmed by colocalization with Methoxy-X04, a known in vivo amyloid targeting fluorophore. Beside possible binding competition with the Vh4H, Methoxy-X04 hampered good validation due to signal cross-over into the red channel. However, colocalization based on the typical autofluorescence patterns of the different Ab deposits resulted in similar findings. Selectivity was confirmed by lack of nonspecific background signal. Although both Vh4H were capable of targeting Ab in vivo, only pa2H-Alexa594 was detectable after two days, mainly bound to vascular amyloid.

Specific in vivo Ab detection by BBB disruption. Based on the above findings, co-injections of pa2H-Alexa594 with mannitol were done in the right carotid artery to selectively open the BBB in the ipsilateral hemisphere to study the in vivo characteristics throughout the brain. Two hours post-injection, fluorescence was detected in the right hemisphere, co-localizing with Ab. (Figure 4) Even within the deeper brain structures, no nonspecific binding was observed. Ab related fluorescent signal remained detectable for at least 24 hours post-injection. Without BBB disruption or within wildtype littermates, no apparent Ab labeling could be detected.

Immunofluorescence using VHH-Alexa594. Selectivity for specific Ab deposits was not altered after fluorescent labeling of the VHH, since on human sections, ni3A-Alexa594 selectively stained vascular Ab (Figure 5 A–C), and pa2H-Alexa594 stained both parenchymal and vascular Ab. (Figure 5 G–I) On murine material all Ab deposits were stained by both fluorescent VHH. (Figure 5 D–F & J–L)

Discussion

In this study, we assessed two previously described VHH for their potential to cross the blood-brain barrier and distinctively detect vascular and parenchymal Ab deposits in vivo. Specific detection of parenchymal and vascular amyloid in APP/PS1 mice

Both Vh4H stained positive for Ab upon APP/PS1 brain sections confirming appropriate use of this transgenic model. In vivo binding to parenchymal and vascular Ab was confirmed when the BBB was circumvented. Signal remained detectable for at least 24 hours while in vivo pa2H showed a high affinity combined with a low off-rate. However, previously shown selectivity for solely vascular Ab in human post-mortem brain sections by ni3A was not
Figure 4. Specific \textit{in vivo} Aβ binding after BBB disruption. After disruption of the BBB using a co-injection of 15% mannitol with pa2H-Alexa594 into the right carotid artery of an aged APP/PS1 mouse sacrificed 2 hrs post injection, amyloid plaques are clearly depicted in both hemispheres using a Thioflavin T (ThT) staining (A), while the pa2H-Alexa594 signal is only detected in the right hemisphere (B). More careful examination shows all Alexa594 signal colocalizes with ThT in the right hemisphere, while in the left only some autofluorescense can be detected. Furthermore, immunofluorescense anti-Aβ staining of the plaques using Alexa488 within the left hemisphere (C) results only in green signal, while within the right hemisphere (D) the red signal from pa2H-Alexa594 nicely colocalizes within the plaques. Experiments performed in a similar setting...
observed within this mouse model (Figures 1, 3, 5). Fluorescent or radiolabeling prior in vivo application did not affect their specificity. The unique specific reactivity of n3A for vascular amyloid deposition on human brain material is not yet completely understood [9]. Known differences in morphology and composition of human and murine Aβ deposits might help to understand n3A’s specific reactivity [21,22]. Human plaques consist of discontinuous patches with decreased density and random fibrillar orientation within the amyloid core; murine plaques are generally built up by long organized fibrils, resulting in densely packed amyloid plaques with a relatively large core [23]. Besides morphological differences, posttranslational modifications of Aβ differ from mouse to man leading to alterations of the Aβ molecule itself [21,24,25]. Differences in metal ion content are known to influence the tertiary structure [26,27]. Previous epitope mapping revealed that n3A has no other cross reaction but to Aβ1–42 [9], which is highly abundant in parenchymal and vascular deposits in both humans and APP/PS1 mice. All together, this leads to the conclusion that the selective reactivity of n3A must depend on the structural presentation of Aβ1–42, in which case murine parenchymal plaques probably show structural similarities to human CAA.

**In vivo blood-brain barrier passage**

Previous in vitro data suggested that our VHH actively migrated across the BBB in a more efficient way than FC5, a VHH specifically selected to pass the BBB [12]. However, the in vivo experiments resulted only in a small cerebral uptake of the tagged 99mTc-pa2H at 24 hours after intravenous administration, and the current brain uptake levels were insufficient to assess the uptake kinetics in vivo with for example SPECT imaging. (data not shown) Additional experiments with untagged DTPA(111In)-pa2H further confirmed the current limitations as hardly any cerebral uptake was observed with this labeling protocol. The increased brain uptake for 99mTc-pa2H compared to 111In-pa2H may be due to the slower blood clearance for 99mTc-pa2H. The observed fast blood clearance and relatively high renal retention for the VH in this study is in line with previous reports [28,29], and

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**Figure 5. Immunofluorescence with VHH-Alexa594.** Shown are the results of immunofluorescence staining with n3A- and pa2H-Alexa594 on cryosections of APP/PS1 murine and human AD/CAA brain tissue, including wildtype or healthy controls. Both VHH stain positive for CAA in all sections (A, D, G, J). Only n3A-Alexa594 stained negative for human parenchymal Aβ (B), while pa2H stained positive for several types of parenchymal Aβ deposits (G, H, J, K) in both humans and mice. In either human or murine control tissue no such staining patterns were observed. (C, F, I, L)

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