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In vitro and in vivo Staining Characteristics of Small, Fluorescent, Aβ42-Binding D-Enantiomeric Peptides in Transgenic AD Mouse Models

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One of the characteristic pathological hallmarks of Alzheimer's disease (AD) are neuritic plaques that consist of amyloid peptide (Aβ). To improve diagnosis and treatment evaluation, neuroimaging tools that make use of Aβ-binding ligands to visualise amyloid plaques are being developed. We investigate the in vitro and in vivo characteristics of a series of three D-enantiomeric peptides (D1–D3) that were developed to specifically bind amyloid β1-42 (Aβ42) in the brains of transgenic AD-model mice. We stained brain sections of the mice, injected and infused the mice with these small D-peptides, and examined their staining of Aβ42 in the brain. The experiments demonstrate that the D-peptides label all plaques that contain Aβ42 in the brain. In contrast, diffuse amyloid β deposits (which do not contain Aβ42) are not stained by any of the D-peptides. The in vivo and in vitro studies demonstrate that the D-peptides label all Aβ42 in the brain, and none of the D-peptides causes inflammation or is taken up by astrocytes or microglia. Furthermore, long-term infusion of the peptides does not cause inflammation. Together, this demonstrates that these D-peptides might be suitable for use as molecular probes to measure Aβ plaque load in the living brain for early diagnosis of Alzheimer's disease, or to monitor Aβ42 plaque load during disease progression or during treatment.

Introduction

In elderly people Alzheimer's disease (AD) is the most common form of dementia.[1] The two pathologies that characterise the disease are the presence of large numbers of intracellular neurofibrillary tangles (NFTs) and extracellular neuritic plaques in the brain.[2–4] Neurofibrillary tangles consist of hyperphosphorylated, twisted filaments of the cytoskeletal protein τ,[5] whereas plaques are primarily made up of amyloid β (Aβ)[4,6] a 39–43 amino acid peptide that is derived from the proteolytic processing of the amyloid precursor protein (APP).[4,7] When APP is sequentially cleaved by β-secretase and γ-secretase, one of the resulting breakdown products is Aβ; in contrast, initial cleavage by α-secretase (in the middle of the Aβ sequence) leads to production of APPs-α and the C83 peptide.[4]

The appearance of the pathological processes probably occurs many years before cognitive symptoms appear.[8,9] Therefore in vivo detection and quantification of amyloid species in the brains of patients during the course of the disease, and for the early diagnosis and evaluation of the effects of AD therapies is an emerging field in AD research. As already shown, the specificity of AD diagnosis can be improved by using glucose-metabolism-sensing positron emission tomography (PET) experiments[10] and perfusion single-photon emission-computed tomography (SPECT).[11] An even better characterisation of amyloid plaque load in the brain can be expected from imaging approaches that make use of amyloid ligands. The development of amyloid-specific ligands started 10 years ago, but many substances failed due to intolerably unspecific binding and poor distribution in the brain in animals. Today, only four amyloid PET ligands have been applied in PET studies (for a review, see Ref. [12]). The most prominent and successful one so far seems to be Pittsburgh compound B (PIB),[13] a benzothiazole derivative that has been described as specifically binding to fibrillar Aβ.

Transgenic mice expressing mutated human AD genes offer a powerful model to study the role of Aβ in the development of pathology.[14,15] We employed small, specific Aβ42-binding D-amino acid peptides to study the specific role of Aβ42 in the development of Alzheimer's disease in vivo.[16,17] In summary, three Aβ42-binding D-peptides were identified by using a mirror-image phage display selection by using Aβ42 as a target. D1, as an example, binds Aβ42 in vitro with a K_d value in the sub-micromolar range and stains Aβ plaques in human brain tissue sections in vitro.[16–18]

We compare the in vitro and in vivo binding characteristics of several D-peptides that were obtained from different mirror-image phage display screening procedures. To do this, we employed three different lines of transgenic mice that express both human APPswe (swe = Swedish mutation) and/or PS1 mutations. These mice develop elevated levels of Aβ42 at dif-

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ferent ages, and at different locations.[19] We show that at least one of the D-peptides, which shows different properties in respect to sensitivity and specificity in vivo might be suitable for being further developed to be used as a molecular probe to monitor Aβ plaque load in the living brain.

Results and Discussion

In vitro (slice) staining

The staining of paraformaldehyde-fixed brain sections of AP/PS and AP/PSΔ mice with the D-peptides revealed that all three FITC-labelled D-peptides (FITC-D1, FITC-D2, FITC-D3) bind to all dense Aβ plaques that contain Aβ42 (Figure 1). Diffuse Aβ deposits, which are especially present in the sections of the AP/PS mice in large amounts, were not stained. Furthermore, very little Aβ was stained in the AP mouse brain sections (not illustrated), but the APP mutation in these mice is within the Aβ sequence and thus leads to Aβ proteins with different sequence and properties. The staining intensity of the Aβ deposits was directly related to both the FITC–D-peptide concentration that was tested (i.e., 0.01, 0.001, and 0.0001 mg mL^{-1}), and to the amount of time that was used for reaching optimal staining density (not illustrated). At the highest concentration the optimal staining time was less than 5 min (i.e., with the best signal-to-noise ratio), whereas at the lowest concentration (0.0001 mg mL^{-1}) the time was more than 6 h. It should be noted that with the lower concentrations, and longer staining time, the amount of nonspecific staining (i.e., background) was significantly decreased. Similarly, post-staining rinsing of the stained sections in buffer decreased the amount of background staining, and even washing for 24 h in buffer did not change the intensity of the specific binding. Moreover, in general, the FITC–D3 peptide gave rise to slightly higher levels of nonspecific staining than the FITC–D1 peptide (Figure 1), but specific binding was higher, as well.

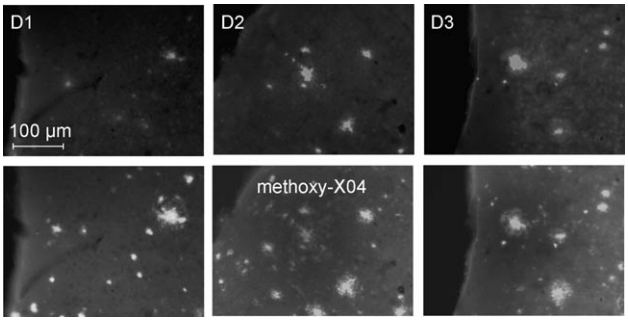

Figure 1. Six photomicrographs of coronal sections of the parietal cortex of a Tg AD-model mouse brain (AP/PS). Top row: fixed brain sections that were stained with FITC-labelled D-peptides (0.001 mg mL^{-1}); bottom row: the same sections stained with methoxy-X04. Note that the background in the D-peptide sections is autofluorescence due to slight overexposure to show tissue. Also note that methoxy-XO4 stains all Aβ in contrast to the D-peptides, which only bind to Aβ42.

Comparison of the FITC–D-peptide stainings with the methoxy-X025 amyloid staining of sections from the AP/PS and AP/PSΔ mouse brains showed that there was nearly complete overlap between the location of methoxy-X04 staining (i.e., plaques) and the staining with the FITC–D-peptides (Figure 1). Due to the decreased fluorescence intensities of the FITC dye in comparison with methoxy-X04, FITC–D-peptide staining appeared less intensely in general. In addition, FITC–D2 showed the least overall staining intensities. Similarly, comparison of the immunohistochemical staining of the adjacent sections for human amyloid (with the W0-2 antibody, which is specific for the human Aβ4–10 sequence) showed that there was complete overlap between the location of dense Aβ staining (i.e., plaques) and the binding of the three FITC–D-peptides (D1–D3), but that the diffuse Aβ deposits were not stained in the AP/PS mouse brain, either in the hippocampus or in the cortex (Figure 2 A).

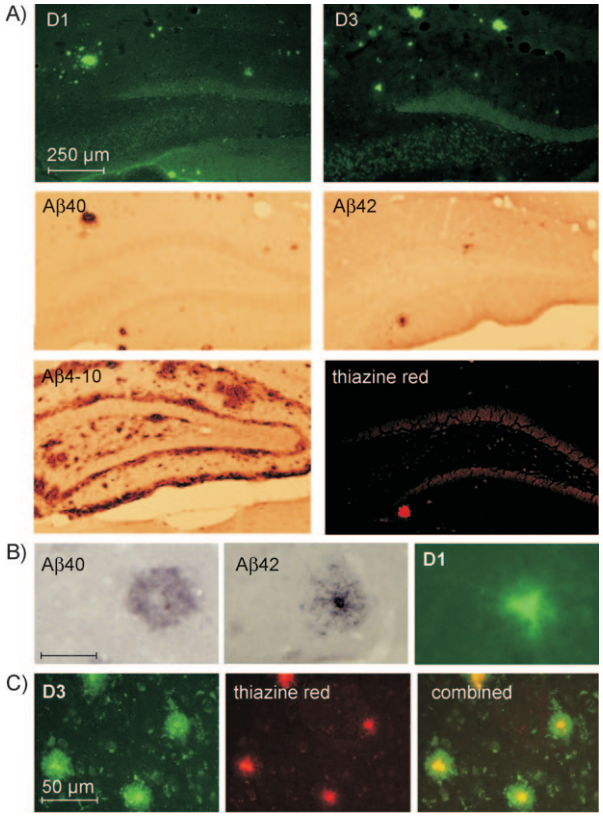

Figure 2. Photomicrographs of coronal sections through the brain of a Tg AD-model mouse (AP/PS) that were stained in vitro with D-peptides (0.0001 mg mL^{-1}). A) Six low-power photomicrographs of adjacent sections of the hippocampus that were stained with different Aβ staining agents. Top row: FITC-labelled D1 and D3; middle row: anti-Aβ40 and anti-Aβ42; bottom row: thiazine red and anti-Aβ4–10. Note the difference between total Aβ staining (i.e., Aβ4–10; all deposits, plaques and diffuse deposits) and Aβ40 and Aβ42 staining (only plaques). B) Three high-power photomicrographs of Aβ plaques that were stained for Aβ40, Aβ42, and FITC-labelled D1, showing the distribution of labelled Aβ. Note that the D-peptide stains similarly to the Aβ42 staining of deposits and not to the staining with Aβ40-specific antibodies. C) Three high-power photomicrographs of the same hippocampal section that had been stained for both FITC-labelled D3 and thiazine red (a marker for proteins with β sheet content), showing the overlap between both plaque core stainings.

Immunohistochemical staining for Aβ40 or Aβ42 of the sections that were adjacent to the sections that were stained with the FITC–D-peptides showed that the distribution of the FITC–D-peptides corresponded more closely to the distribution of Aβ42 than to the distribution of Aβ40 labelling. Both anti-Aβ42 antibody and the FITC–D-peptides stained predominantly the core of the plaques, whereas anti-Aβ40 stained mainly the outside of the plaques (Figure 2B). Sections that were double stained with both anti-Aβ42 and the FITC–D-peptides, demonstrated a total overlap between the site of dense Aβ42 deposits and the location of the three FITC–D-peptides. Furthermore, staining with β-sheet markers such as thioflavine S, Congo red, or thiazine red revealed that all Aβ deposits with a β-sheet-positive core were also stained with all three FITC–D-peptides (Figure 2C).

To analyse whether the binding characteristics of FITC–D1 are dependent on its amino acid sequence or rather on its amino acid composition in sum, a scrambled (sequence) peptide (i.e., sc-D1) was tested. We used both 0.001 and 0.0001 mg mL^{-1} concentrations of FITC-labelled sc-D1 on fixed brain sections of elderly (18 months of age) AP/PS mice. In contrast to FITC–D1, FITC–D2, and FITC–D3, the FITC–sc-D1 peptide showed significantly decreased binding to Aβ in any type of amyloid deposit (Figure 3); this indicates that FITC–D1

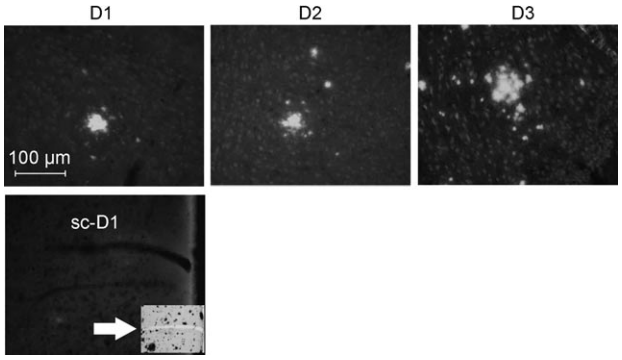

Figure 3. Four high-power photomicrographs of coronal sections through the parietal cortex of an 18-month-old AP/PS mouse. The top three photomicrographs demonstrate the typical staining of plaques with FITC-labelled D1, D2, and D3 as indicated. The lower photomicrograph shows the staining of FITC-labelled sc-D1. The insert in the sc-D1 picture is the adjacent section stained for Aβ. All sections were stained with the same concentration of peptide (0.0001 mg mL^{-1}) and for the same amount of time (1 h); note the lack of staining by the scrambled FITC-labelled D-peptide (sc-D1), lightly stained plaque core indicated by arrow.

binding to Aβ42 is specific for its sequence and cannot be attributed, for example, to sole electrostatic interactions with Aβ.

In these binding experiments all peptides that were used had been conjugated with a FITC molecule for visualisation purposes, therefore we tested whether D1 would show distinct binding characteristics when it was conjugated with different fluorophores in a final set of experiments, that is, we wanted to study the interaction of the fluorescent moiety with the Aβ binding. The data show that no differences in specific binding are present at the 0.001 and 0.0001 mg mL^{-1} concentrations between these D-peptides (Figure 4). The D1 that was conjugated to Oregon green, which is similar in size and charge to FITC, bound Aβ42 similarly to the FITC–D1, but the Bodipy–D1 (Bodipy is smaller and more polar than FITC) showed significantly increased background staining (Figure 4) in both the AP/PS and AP/PSΔ mouse brain sections.

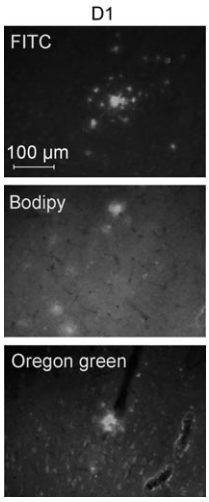

Figure 4. Three photomicrographs of coronal sections of the parietal cortex of an 18-month-old APP/PS1 mouse that were stained with D1 that had been conjugated with different fluorophores, showing staining with D1–FITC, D1–Bodipy, and D1–Oregon green, as indicated. All sections were stained with the same concentration of peptide (0.0001 mg mL^{-1}) and for the same amount of time (1 h). Note the increased background staining with D1–Bodipy, as well as the similarity of staining between D1–FITC and D1–Oregon green.

In vivo staining

The injection of the three D-peptides did not change any obvious physiological parameters (e.g., general health or growth as measured by body weight) in the injected AP/PS mice, nor did it cause any apparent discomfort. Furthermore, none of the animals showed any signs of inflammation in the brain other than that caused by the trauma of the needle penetration (Figure 5).

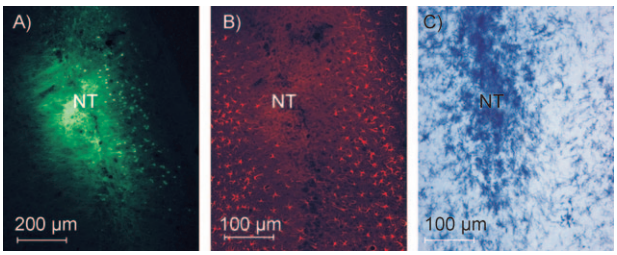

Figure 5. One set of three photomicrographs of adjacent coronal sections through the brain of a non-Tg mouse that was injected with the D1 peptide two days before it was sacrificed. A) Photomicrograph of an unstained section of the entorhinal cortex showing the D1–FITC labeling. B) Photomicrograph of the adjacent section stained for GFAP (astrocytes, red). C) Photomicrograph of the adjacent section, which was stained for CD11b (microglia). Note that the only inflammation that is present is at the needle tract (NT).

Two days or one week after the injection, the three injected groups of mice were sacrificed and assessed for pathology. FITC–D-peptides were injected in the entorhinal cortex in all animals. There was no significant difference in the labelling pattern or brightness of the labelling between both time points. Analysis of the sections that were stained for GFAP or microglia revealed that the injections did not cause any significant inflammation other than that associated with the physical trauma of the injection (Figure 5).

The extent of the distribution of the infused peptide in the brain was analysed in the non-counterstained sections of the brain (a FITC molecule had been conjugated to the injected D-peptides). Inspection of brain sections with a fluorescent microscope revealed labelling of dense Aβ deposits throughout the brain, with maximum labelling brightness near the injection site and a decrease in brightness going outward from the injection site. It should be noted that, the diffuse Aβ deposits in the brain were not labelled; this is similar to the in vitro staining results. Furthermore, the deposits in the FITC–D3-injected mice were brighter than similarly located deposits (i.e., at the same distance from the injection site) in either the FITC–D1 or FITC–D2-injected mice. Finally, whereas all FITC–D-peptides only labelled dense Aβ deposits, FITC–D3 peptide was taken up by some neurons, predominantly near the injection site (Figure 6A). The FITC–D3-labeled neurons show clearly

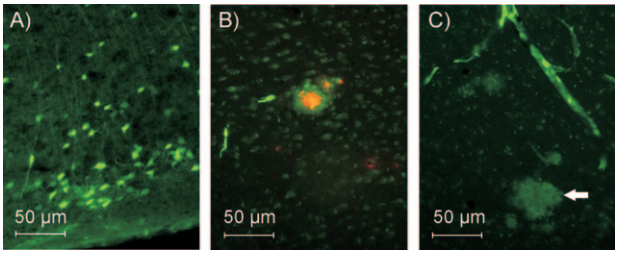

Figure 6. One set of photomicrographs of FITC–D3 injections in non-Tg and Tg AD-model mice. A) Photomicrograph of the brain of a non-Tg mouse that was injected with FITC–D3 showing an unstained section of the entorhinal cortex; this demonstrates labelling of neurons with FITC–D3, B) photomicrograph of a thiazine-red-counterstained section of the entorhinal cortex of a Tg AD-model mouse that had been injected with FITC–D3, showing D3-labelled plaques with a thiazine red core; C) photomicrograph of an unstained section of the piriform cortex of a Tg AD-model mouse that had been injected with FITC–D3. This demonstrates the labeling of perivascular cells around the blood vessels and light labelling of plaques (arrow).

labelled dendrites and axons (Figure 6B). It should be noted that no glial cells (either astrocytes or microglial cells) showed any uptake of the FITC–D-peptides at any time. An exception to this rule were pericytes/perivascular macrophages that showed uptake of FITC–D3 throughout the brain (Figure 6C).

An infusion cannula was implanted in the dorsal hippocampus in all AP/PSΔ animals. Analysis of the sections that were stained for GFAP or microglia revealed that the infusions did not cause any significant inflammation or pathology. The only inflammation that was present was around the infusion cannula itself, and there was no difference in inflammation between the saline and peptide-infused animals. The extent of the distribution of the infused peptide in the brain was analysed in the unstained sections of the brain because 10% of the infused D-peptide was conjugated to a FITC molecule. Inspection of brain sections with a fluorescent microscope revealed that all dense Aβ deposits in the whole brain were labelled, with a decrease in brightness further from the infusion site. Additionally, the deposits in the D3-infused mice were brighter than similarly located deposits (i.e., at the same distance from the infusion site) relative to the D1-infused mice. Similar to the brain injections, D1 was only present at Aβ deposits, but a small amount of D3 had also been taken up by neurons. These neurons were predominantly present near the infusion site, and furthermore, pericytes/perivacular macrophages throughout the brain showed labelling with D3 (Figure 7). It should be

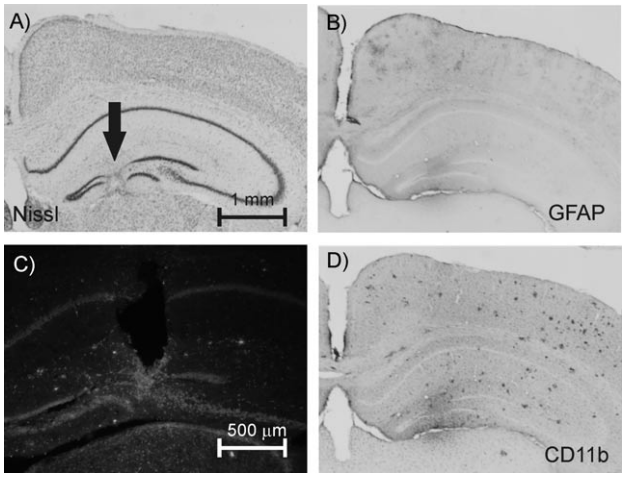

Figure 7. One set of four photomicrographs of adjacent coronal sections through the brain of an AP/PSΔ mouse that was infused with the D3 peptide for four weeks. A) photomicrograph of a Nissl-stained section of the dorsal cortex, showing the infusion site (arrow), B) photomicrograph of the adjacent section stained for GFAP (astrocytes), C) an adjacent unstained section showing the infusion of the D3–FITC, and D) photomicrograph of the adjacent section stained for CD11b (microglia). Please note that the only inflammation present is at the needle tract (NT).

noted that neither the infusion site area, nor anywhere else in the brain, including any glial cells (astrocytes and microglia) showed any signs of uptake of the D-peptides.

Conclusions

We investigated the in vitro and in vivo binding characteristics of three small D-amino acid peptides (i.e., D1, D2 and D3) that were developed to specifically bind Aβ42.[16,18] For visualisation purposes, in parts of the study the peptides were FITC labelled. We examined the labelling of Aβ deposits in transgenic AD-model mouse brains, in the hippocampus and cortex. The data demonstrate that all dense Aβ deposits (plaques) that contain Aβ42 are labelled with the D-peptides, but not diffuse deposits, and the blood vessel walls are only stained lightly (at ages when these deposits contain detectable Aβ42, data not

shown). This corresponds to the distribution of Aβ42 in the brain as visualised by Aβ42 specific antibodies.

In brain tissue sections that were derived from AD patients, amyloid plaques and leptomeningeal vessels that contain Aβ42 are stained positively with the fluorescence-labelled derivative of D1.[16] In contrast, fibrillar deposits that are derived from other amyloidosis are not labelled by D1.[16] None of the D-peptides showed any binding to Aβ42 deposits in the brains of mice that express the APPswe/dutch/iowa mutation. This underlines the high specificity of all these D-peptides for wild-type Aβ42 because the Dutch and Iowa mutations[20–23] are within the Aβ peptide sequence of APP, in contrast to the Swedish mutation, which is outside the Aβ sequence, and only influences Aβ processing.[4]

The data demonstrate that none of the three D-peptides binds to diffuse Aβ deposits, but they do bind to dense Aβ deposits (i.e., plaques). Earlier we have shown that the diffuse Aβ deposits do not stain with thioflavine S, Congo red or thiazine red, but dense deposits (i.e., plaque cores) do. Furthermore, the diffuse deposits consist primarily of N-terminal fragments of Aβ; they contain some Aβ40 but do not contain stainable amounts of Aβ42,[24] in contrast to plaques that consist of significant amounts of both Aβ40 and Aβ42. We have suggested earlier that the diffuse deposits consist of Aβ that has a different length (and structure) from the Aβ42 and Aβ40 that are present in plaques, even if Aβ fibrils are present in the diffuse deposits.[24] Together these data indicate that the D-peptides bind very specifically to only Aβ42.

In previous experiments, we and others have shown that APP and Aβ are axonally transported.[24–27] Similarly, in the animals that were injected with D3 in the entorhinal cortex, neurons in layer II of the entorhinal cortex showed uptake of D3, and small amounts of D3 were present at the terminal fields of these neurons. Furthermore, neurons in the entorhinal cortex were labelled after D3 infusion into the hippocampus. It has been suggested that Aβ42 is taken up by neurons after binding to the α7 nicotinic acetylcholine receptor, and that this receptor is especially prominent in the entorhinal cortex.[28,29] These neurons, however, did not show any sign of taking up D2 after injections. This different behaviour among the D-peptides might be due to the slightly different structure and binding characteristics. These results demonstrate that D3 is taken up by neurons near the injection/infusion site, and only D3 is axonally transported in a manner similar to normal Aβ.[30] It has been suggested that the axonal transport of Aβ is essential for normal synaptic functioning.[26] These D3-specific observations could be explained with a significant affinity of D3 to monomeric Aβ or APP. Upon binding to Aβ or APP, it would be transported within the cell and become indistinguishable from its target.

Plaques in AD patients and in AD-model mice are quite often accompanied by activated glial cells, both astrocytes and microglia,[31,32] and it has been suggested that especially Aβ42 has inflammatory properties.[33] In contrast, neither the injections nor infusions of the D-peptides caused any inflammation. This is consistent with the much lower immunogenic properties of D-peptides relative to L-peptides, in general.[34,35]

Together, we have demonstrated that 1) these D-peptides specifically bind to Aβ42 in the brains of APP/PS1 transgenic mice, and 2) that injections or infusions of the D-peptides do not cause an inflammatory response. Thus, our data strongly suggest that these novel and highly specific Aβ42 ligands, especially D1, have potential application(s) in the diagnosis and therapy of Alzheimer's disease, especially because these D-peptides are much more resistant to proteolysis than natural L-peptides. Among all three tested D-peptides, D1 seems to have the highest potential to be used for in vivo imaging because D2 stained Aβ less intensively, and D3 was taken up by neurons. The latter finding surely deserves more thorough investigation. As with any other substance to be used as a probe for in vivo imaging, D1 or any derivative thereof cannot be applied directly into the brain, further studies are needed to determine to what extent D1 is able to cross the blood–brain barrier. The first studies enlightening the D1 biodistribution in animals, including intravenous application and nasal treatment are encouraging. In addition, because D-enantiomeric peptides are fully synthetic, any desired modification to improve penetration properties can easily be done, if necessary.

Experimental Section

Animals

Two lines of APP and PS1 single and double transgenic mice ($n=48$) were used. The first line of mice was generated from matings between APPswe and HuPS1 A246E transgenic mice (AP/PS), this mouse line was originally produced at the Johns Hopkins University (Baltimore, MD, USA),[36] and was bred locally on a C57BL/6 J background. The second line of APP/PS1 mice was the APPswe+PS1Δ9 line (AP/PSΔ).[36] The mice were acquired from JAX (The Jackson Laboratory, Bar Harbor, ME, USA) at the age of six weeks. The third line of transgenic mice is the APPswe/dutch/iowa line (AP), these mice were originally produced at Stony Brook University (Stony Brook, NY, USA)[37] and were bred locally for this study. The animals were housed four to a cage in our facility, in a controlled environment ($T=22\,^{\circ}C$, humidity 50–60%, light from 07:00–19:00), with food and water available ad libitum. All procedures were conducted in accordance with the local Institutional Animal Care and Use Committee (IACUC) guidelines.

Peptides

A mirror-image phage display approach was used to identify novel and highly specific ligands for Alzheimer's disease amyloid peptide Aβ1–42.[16] In short, a randomised 12-mer peptide library presented on M13 phages was screened for peptides with binding affinity for the mirror image of Aβ1–42.[16] For the first phage display selection procedure, biotinylated bio-D-Aβ(1–42) was dissolved in hexafluoroisopropanol (HFIP) to a concentration of 2 mM and further diluted to 250 μM in dimethyl sulfoxide (DMSO). The solution was mixed in a ratio of 1:1000 with TBST (50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5, 150 mM NaCl, 0.1% (*v*/*v*) Tween-20) that contained bovine serum albumin (BSA, 0.1% *w*/*w*) and 1×1011 phages (PhD-12 Phage Display Peptide Library Kit, New England Biolabs, Frankfurt, Germany). The final concentration of bio-D-Aβ-(1–42) was 250 nM. After 10 min, the phage–peptide suspension was transferred into a streptavidin-coated tube (Roche–Boehringer, Mannheim, Germany) and gently shaken for 15 min at room tem-

perature. To displace any streptavidin-binding phages, biotin was added to a concentration of 0.1 mM. Nonbinding phages were discarded by washing the tubes with TBST (10×). The phages were eluted by incubation with 0.2 mM glycine-HCl (pH 2.2) for 10 min. The solution was neutralised with 1 M Tris-HCl (pH 9.1). The eluted phages were amplified to yield 1×1011 phages to be used as input for the next selection round, as described in the standard protocol by New England Biolabs (PhD-12 Phage Display Peptide Library Kit Manual, New England Biolabs, Frankfurt, Germany). After four rounds of selection and amplification, the peptides were enriched to yield a dominating consensus sequence. The mirror image of the most representative peptide (i.e., D1) was shown to bind Aβ42 with a dissociation constant in the sub-micromolar range.[16] The D2 peptide was derived from another phage display selection against Aβ. The conditions for this approach were similar to those for the selection of D1. In selection 2 (resulting in D2) Aβ was dissolved in DMSO instead of HFIP and then diluted in HFIP similar to selection 1. In both selections, Aβ was further diluted in TBST to a final concentration of 250 nM. For the third phage display selection procedure, D-Aβ(1–42) was dissolved in HFIP to a concentration of 20 μM and diluted 1:10000 in TBS (Tris-HCl, pH 7.5, 150 mM NaCl). The final concentration of D-Aβ(1–42) was 2 nM. This solution was immediately transferred into a streptavidin-coated tube (Roche–Boehringer, Mannheim, Germany) and gently shaken for 15 min at room temperature. The tubes were washed with TBS (2×) and stored at −20 °C. For the selection procedure, 1×1011 phages (PhD-12 Phage Display Peptide Library Kit, New England Biolabs, Frankfurt, Germany) were diluted in TBST with 0.1% BSA (*w/w*) and transferred into one of the prepared tubes. After 10 min incubation at room temperature, the tubes were washed with TBST (10×) and the elution of the binding phages was executed as described above. The mirror image of the most representative peptide from the selection was D3.

D1 (QSHYRHISPQV), D2 (GISWQQSHHLVA), D3 (RPRTRLHTHRNR), and scD1 (scrambled D1 sequence, HSSPQIVHQAYR) peptides were purchased as synthetic D-enantiomeric peptides (JPT, Berlin, Germany). For visualisation purposes, in most binding experiments peptides were used with a FITC molecule that was conjugated via the ε-amino group of a C-terminally added lysine residue. In a few experiments, D1 was conjugated with other fluorophores to study the interaction of the fluorescent moiety with binding characteristics of the D1 peptide.

Entorhinal cortex injection

In three groups of eight-month-old AP/PS mice, we unilaterally injected the entorhinal cortex with one of the D-peptides, one group with the D1 peptide ($n=6$), one group ($n=6$) with the D3 peptide, and one group with the D2 peptide ($n=4$). The peptide concentration was 0.05 mg in 50 μL, the D-peptides were conjugated to a FITC molecule (to be able to visualise the injection and binding). In short, mice were anaesthetised, placed in a stereotactic frame, a hole was drilled above the right entorhinal cortex, and the needle of a Hamilton syringe (90 μm diameter) was lowered into the entorhinal cortex. The solution of the respective D-peptide in saline (pH 7.4) was unilaterally pressure injected (250 nL) into the entorhinal cortex at a rate of 50 nL min^{-1}. Following the injection, the needle was left in place for another 5 min before retraction. Two days or one week after the injections were made, the animals were sacrificed for histopathological analysis (see below).

Hippocampal infusion

In three groups of eight-month-old AP/PSΔ mice ($n=12$), we unilaterally infused the hippocampus for four weeks by using Alzet osmotic minipumps, one group ($n=4$) with saline, one group with the D1 peptide ($n=4$), and one group with the D3 peptide ($n=4$). The peptide concentration was 0.25 mg pump^{-1}, that is, 0.25 mg in 250 μL of the 0.25 mg peptide, 0.225 mg was unconjugated peptide, 0.025 mg was peptide that was conjugated with a FITC molecule (to be able to visualise the infusion). The Alzet minipump (model #2004; delivery rate: 0.25 μL h^{-1}; duration: 4 weeks; Alzet, Cupertino, CA, USA) was filled with the appropriate solution, and they were connected to the cannula, such that no air bubbles were present. Then the cannula (Alzet Brain Infusion Kit 3, Alzet) were implanted in the brain (right dorsal hippocampus). In short, the mice were anaesthetised, placed in a stereotactic frame, a hole was drilled above the right dorsal hippocampus, and the cannula was lowered into the hippocampus. Four weeks after the implantations the animals were sacrificed for histopathological analysis.

Histopathology

In short, the mice were anaesthetised, transcardially perfused with saline followed by 4% paraformaldehyde and the brains were removed from the skull. After fixation (4 h) and cryoprotection (24 h in 30% sucrose), six series (1 in 6) of coronal sections were cut through the brain. The first series of sections was mounted unstained, and the second, third and fourth series were stained immunohistochemically according to published protocols.[19,38] The other two series were stored in at −20 °C in antifreeze for future analysis. One half of the second series was stained for human Aβ by using the W0–2 antibody (mouse anti-human Aβ4–9[39]), the other half of the second series was stained for mouse Aβ (rabbit anti-rodent Aβ; Covance, Princeton, NJ, USA).[19] The first half of the third series was stained for Aβ40 (mouse anti-Aβ40, Covance) the other half for Aβ42 (mouse anti-Aβ42; Covance). In some animals, one half of the fourth series was stained for GFAP (mouse anti-GFAP; Sigma), whereas the other half was stained for CD11b (rat anti-mouseCD11b; Serotec, Oxford, UK), which is a marker of microglia to analyse inflammation in the brain. Some of these sections were double stained with either Congo red, thioflavine S or thiazine red to visualise β sheets (i.e., Aβ plaque cores). In a few animals methoxy-X04[39] was infused during the perfusion to label all of the Aβ in the brain. The sections that were destined for immunohistochemical Aβ staining were pretreated for 30 min with hot (85 °C) citrate buffer. The series of sections were transferred to a solution that contained the primary antibody (W0–2, mouse monoclonal), this solution consists of TBS with 0.5% Triton X-100 (TBS-T). Following incubation in this solution for 18 h on a shaker table at room temperature (20 °C) in the dark, the sections were rinsed in TBS-T (3×) and transferred to the solution that contained the appropriate secondary antibody (goat anti-mouse–biotin; Sigma). After two hours, the sections were rinsed with TBS-T (3×) and transferred to a solution that contained mouse ExtrAvidin (Sigma); after rinsing, the sections were incubated for approximately 3 min with Ni-enhanced DAB.[38] In a small number of sections, the Aβ deposits were double labelled for Aβ40 and Aβ42 by using fluorescent secondary antibodies. All stained sections were mounted on slides with cover slips. It should be noted that the other Aβ and APP antibodies were used by following a similar protocol with the appropriate secondary antibodies.

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