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Reduction of Alzheimer's Disease Amyloid Plaque Load in Transgenic Mice by D3, a D-Enantiomeric Peptide Identified by Mirror Image Phage Display

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Alzheimer's disease (AD) is a multifactorial disorder, which is characterized by progressive memory deficits, cognitive impairments and personality changes. More than 20 million people are affected worldwide.[1] The histopathological hallmarks of AD are aggregated protein deposits (i.e., senile plaques and neurofibrillary tangles) in the brain. Senile plaques consist mainly of extracellular amyloid-β peptide (Aβ) deposits. While there is still debated over whether Aβ is the causative agent in AD, the inhibition of Aβ production and aggregation is often targeted for therapy development.

Recently, we used mirror image phage display to identify a novel D-amino acid peptide binding specifically to Aβ(1–42) with a binding affinity in the submicromolar dissociation constant range and called it "D-pep" or "D1".[2,3] D-peptides are known to be extremely protease resistant and less immunogenic than their respective L-enantiomers,[4] thus being more suitable for in vivo use. D-Peptides have previously been used as inhibitors of amyloid formation to prevent the associated Aβ cytotoxicity.[5–7] Recently, another strategy to obtain D-peptides specifically interacting with amyloid stretches, inhibiting amyloid formation and cell toxicity, was presented.[8]

In the present work, we identified a novel D-enantiomeric amino acid peptide "D3" which might provide a novel basis for therapeutic and preventive approaches to AD. D3 might also be useful as a tool to study the role of Aβ plaques in AD progression. We performed phage display selections of a peptide library encoding more than 1×10^9 randomly different 12-amino acid sequences with D-enantiomeric Aβ(1–42) (D-Aβ) as the target. D-Aβ was dissolved to obtain a low final concentration of 2 nM. Under those conditions, we expected monomeric Aβ or small Aβ oligomers to be the dominant target species during the phage display screening. After 6 rounds of biopanning, we determined the peptide sequences of the enriched phage displayed peptides by DNA sequence analysis of the respective genome region. The dominant peptide sequence obtained from the selection was RPRTRLHTHRNR, referred to as D3. This sequence was found in 9 out of 23 randomly chosen phage clones. Additionally, 9 of the selected peptides were related to the dominating peptide by at least 9 amino acids (table S1, Supporting Information).

We investigated the influence of D3 on Aβ aggregation. The content of amyloid fibrils was determined by Thioflavin T (ThT) fluorescence upon incubation of various Aβ/D3 mixtures. ThT is a benzothiazole dye that exhibits enhanced fluorescence upon binding to amyloid fibrils, and is commonly used to detect amyloid fibrils.[9] ThT fluorescence of Aβ mixtures with D3 was significantly lower than those without D3 (Figure 1a). These results suggest that D3 significantly decreased the formation of ThT-positive Aβ aggregates.

These results were confirmed using a fluorescence correlation spectroscopy (FCS) based assay. FCS allows the detection of Aβ aggregates in highly dilute samples, with concentrations in the nanomolar range.[10] Fluorescence fluctuations generated by single molecules passing the confocal volume of the focused laser beam are measured, and can be evaluated by autocorrelation to obtain the diffusion time of the studied molecules. When Aβ aggregates, containing at least one molecule of Oregon Green (OG) labeled Aβ, are present within a given solution, they can be directly detected as spikes in the fluorescence fluctuation recordings. Due to their size, they have an increased duration of stay in the confocal volume and increased fluorescence intensities because most or all of them contain more than one fluorescence label.[3]

FCS measurements were carried out with 5 nM OG-labeled Aβ in the absence or presence of D3 in various concentrations. Figure 1b shows how the number of peaks decreases with increasing amounts of added D3. A 50% inhibition of aggregate formation by D3 is obtained at ~1 μM. This result suggests that D3 prevents aggregation of Aβ in the nanomolar concentration range.

To assay the ability of D3 to dissolve pre-existing ThT positive Aβ aggregates, Aβ was preincubated without D3 for seven days to allow aggregation. Then, D3 at various concentrations was added and ThT fluorescence was followed (Figure 1c). The results clearly show a dose-dependent aggregate disassembly activity of D3 for preformed ThT positive Aβ aggregates without stirring, ultrasonic treatment, or any other mechanical support.

We studied the effect of D3 on Aβ-induced cytotoxicity in rat pheochromocytoma (PC12) cells. Aβ (10 μM) was incubated without or with varying concentrations of D3 for 6 days at 37 °C. Cells were then treated with various Aβ/D3 mixtures and their viability was measured by MTT reduction (Figure 1d). In the presence of 2 μM of Aβ cell viability dropped to 40%, this effect was reversed in a dose-dependant manner by the addition of D3, and cell viability could be completely rescued in

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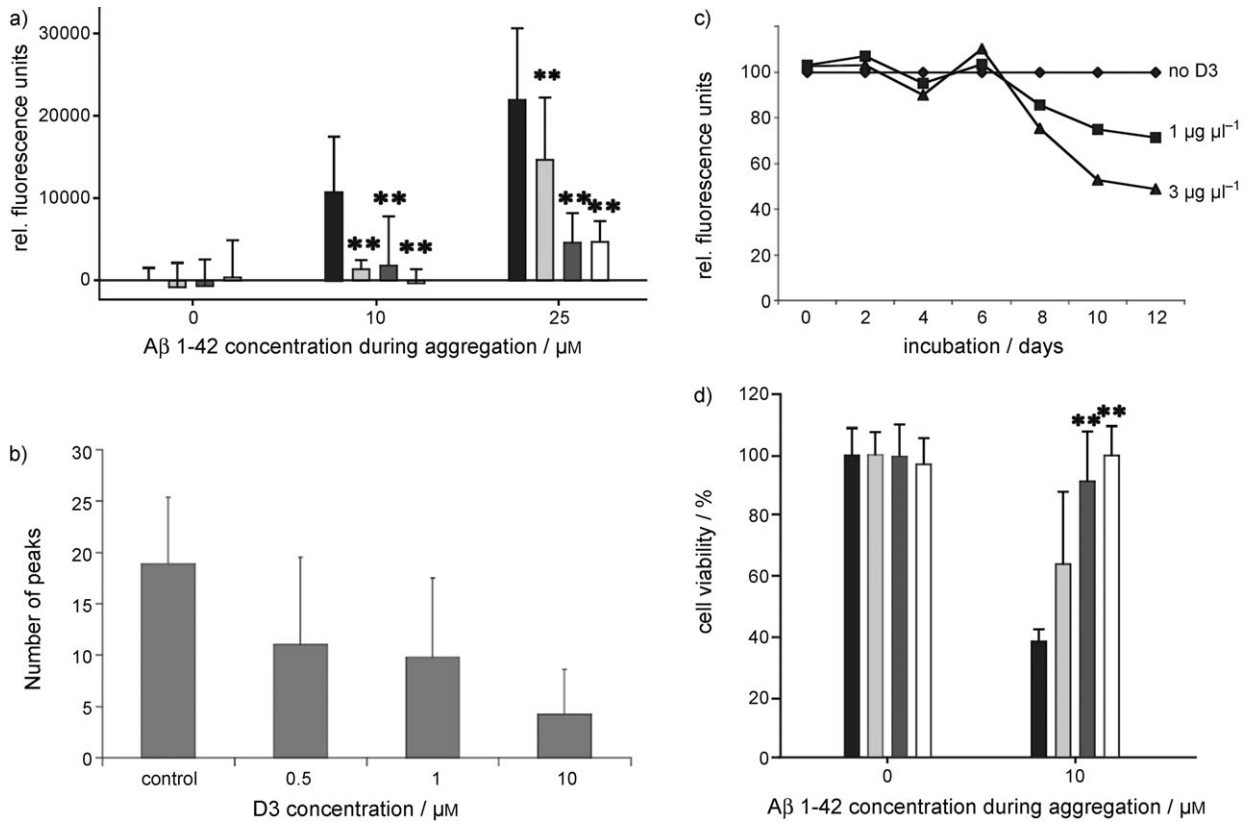

Figure 1. Influences of D3 on Aβ aggregate formation and cell cytotoxicity: a) Inhibition of Aβ fibrillogenesis. D3 was added in concentrations of: 0 (■), 10 μM (■), 100 μM (■), and 1 mM peptide (□) to Aβ (10 or 25 μM). The Aβ/D3-mixtures were incubated for 6 days at 37 °C. Upon addition of Thioflavin T (ThT), fluorescence was measured at 490 nm in relative units (mean +/- standard deviations of results, four separate runs, three replicates per run. * $p < 0.01$; ** $p < 0.0005$, students t-test). b) Aβ aggregate formation in the presence of D3 as measured by FCS. 5 nM OG–Aβ were incubated with or without D3 in PBS with 2% DMSO at RT. Each bar represents the averaged value of four independent measurements. For the controls eight measurements were averaged. c) D3 concentration-dependent dissolution of preformed fibrils quantified by ThT assay. The average of three different samples as a percentage of the amount obtained before adding D3 ($t = 0$) is shown. d) PC12 cell viability in absence or presence of Aβ and/or D3. Different concentrations of D3 (as in Figure 1a) were added to Aβ (10 μM) or to samples without Aβ (controls). The Aβ/D3 mixtures were incubated for 6 days at 37 °C and diluted into PC12 cell cultures (1:5). Cell viability was measured using MTT assay.

the presence of 200 μM D3. Additionally, the associated cytotoxicity of D3 was measured and found not to be toxic up to a concentration of 200 μM.

To assess potential in vivo effects of D3 on amyloid deposition in a living brain, we infused APP and PS1 double transgenic mice with D3 for 30 days. Mice were unilaterally infused in the hippocampus with either saline ($n = 10$), D1 as a peptide control ($n = 9$), or D3 peptide ($n = 10$). Histopathological analysis revealed that all dense Aβ deposits in the brain were FITC-labeled, with a slight decrease in brightness further from the infusion site (Figure 2). All Aβ deposits with a Congo red positive core were found to be FITC-labeled, but neither diffuse Aβ deposits nor Aβ deposits in blood vessel walls were FITC-labeled, none of the control infused mice showed labeling of any Aβ deposits. The distribution behavior of D3 and other D-peptides is reported in more detail elsewhere.[11] The Aβ load in the hippocampus and frontal cortex was measured; significant reduction in the Aβ load was observed in the group infused with D3, both ipsilateral and contralateral to the infusion, compared to the control-infused and the D1 infused mice (Figure 3). In contrast, there was no significant difference between the control and the D1 infused mice in any brain area (Table 1).

Analysis of sections that were stained for GFAP (astrocytes) or CD11b (microglia) revealed that the infusions did not cause any significant inflammation or pathology. Inflammation was only present around the infusion cannula. At first sight, there was no obvious difference in the amount of total inflammation between the control and peptide infused mice. However, a more detailed analysis of the inflammation (i.e., density of activated astrocytes and microglia) around Aβ deposits revealed that the D3 treatment significantly reduced the amount of inflammation markers near to the remaining plaques. The number of activated astrocytes and microglia (measured as density of GFAP and CD11b staining) near plaques of equal size was significantly reduced relative to the control groups (Figure 4), indicating that D3 changes the inflammatory properties of Aβ.

The most intriguing property of D3, however, is its ability to drastically reduce the Aβ plaque load of transgenic mice after a four week treatment with only 9 μg D3 per day per mouse. Previously, antiaggregation agents have been shown to inhib-

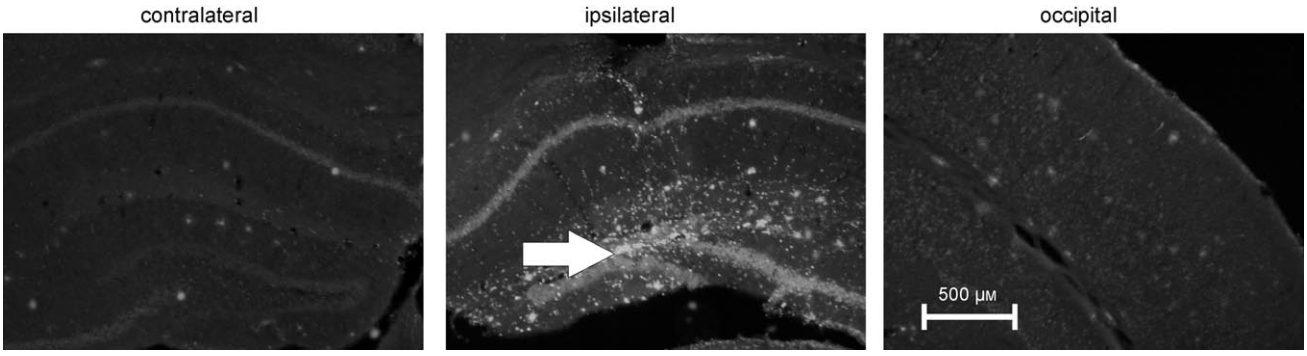

Figure 2. Photomicrographs of different sections through the brain of a Tg AD model mouse (AP/PS), showing the distribution of D3–FITC labeled Aβ via fluorescence microscopy. All dense Aβ deposits in the brain were FITC-labeled, with a decrease in brightness further from the infusion site, indicated by an arrow.

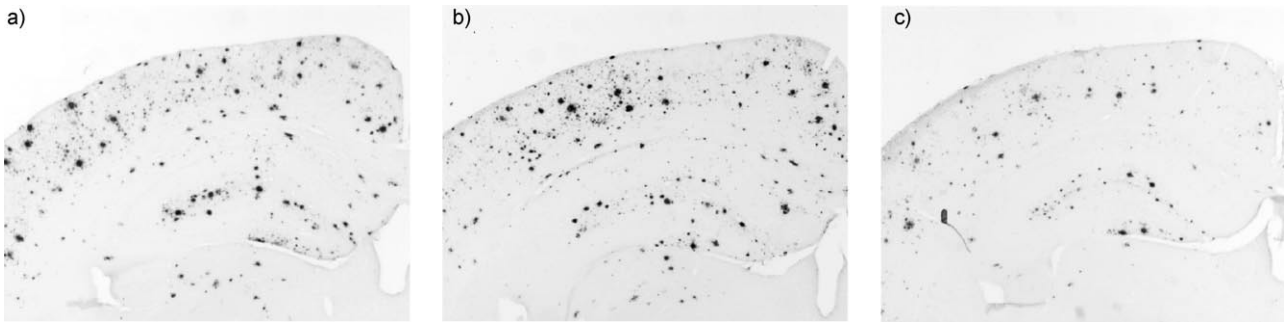

Figure 3. Influences of D3 on Aβ plaque load in brain tissue sections of transgenic APP-PSΔ mice. a) Saline, b) D1 (peptide control) or c) D3 was infused in the brains of mice for 30 days. Brain sections were stained with W0-2 (anti human amyloid β). Representative sections showing the hippocampus and dorsal cortex are shown. Notably, a decrease in Aβ staining in the D3 infused brain compared to the control can be seen.

Table 1. Overview of in vivo experiment results.[a]

Group	Control	D1	D3
Infusion	saline	D1 peptide	D3 peptide
Number[b]	n = 10	n = 9	n = 10
Body weight	34.7 ± 1.4 g	35.1 ± 3.5 g	34.7 ± 1.8 g
Aβ load[c]	2.1 ± 0.4%	2.1 ± 0.2%	1.4 ± 0.1%*
Congo Red	31 ± 5	37 ± 5	22 ± 4*
GFAP[d]	102 ± 2	101 ± 2	89 ± 3*
Cd11b[d]	159 ± 3	165 ± 3	147 ± 2*

[a] * indicates $p < 0.05$. All density measurements were done in triplicate. Data were analyzed by Student's paired t test (ipsi- versus contralateral) and by ANOVA (Systat 11; between groups). [b] Number of animals per group. [c] Number of Congo Red positive plaques. [d] Density of GFAP and CD11b staining around plaques in the dorsal hippocampus.

it/reverse Aβ aggregation in vitro and in the brains of transgenic APP mice.[12,13] Soto and colleagues[14] designed a pentapeptide based on the central hydrophobic region in the N-terminal domain of Aβ acting as a β-sheet breaker. iAβ5 application (2.5 mg compound intracerebroventricular over 2 month) is claimed to reduce the size of Aβ deposits in the brains of rats up to 62%, and up to 67% in six to seven months old transgenic mice. In contrast, for a 40% reduction of amyloid plaques in our study, a one-month treatment with only 9 µg D3 per day per mouse was sufficient.

Currently, we can only speculate about the mechanism by which D3 induces Aβ plaque reduction in the brains of transgenic AD mice. The observed in vitro activities indicate that D3 inhibits Aβ aggregation, and redissolves pre-existing Aβ fibrils; D3 might also disaggregate amyloid plaques in the brain, leading to increased amounts of monomeric Aβ, which is more easily cleared from the brain.[15,16] In our studies, clear FITC fluorescence was present in pericytes, indicating that these cells might have taken up the Aβ–D3 complex. These cells might have helped in the clearance of the complex from the brain through the blood–brain barrier.

When speculating about the mechanism, however, one has to keep in mind that the applied in vitro concentrations (µM to mM) and the in vivo dose (9 µg in about 0.25 mL total brain volume) of both Aβ and D3 are very different. Thus, any conclusion from in vitro data about observed in vivo effects have to be taken with all necessary precaution.

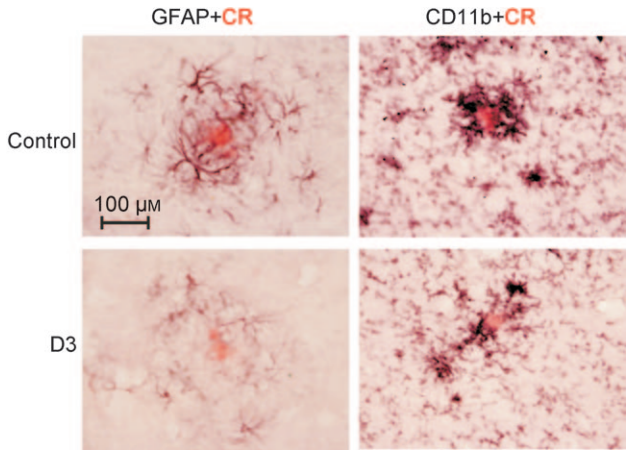

Figure 4. Influences of D3 on inflammation in brain tissue sections of transgenic APP-PSΔ mice. Saline (Control) or D3 was infused in the brains of mice for four weeks. Brain sections were stained with GFAP or CD11b. Representative sections showing Congo red positive plaques in the dorsal cortex are shown. Notably, a decrease in inflammation (activated astrocytes and microglia) surrounding Congo red-positive plaques in the D3 infused brain compared to the control can be seen.

A second possibility could be that D3 binds to Aβ aggregates and marks them as a target for activated astrocytes or microglial cells, which in turn induces increased turnover and clearance processes. However, while the role of microglia in AD is controversial,[17] the observed decrease in active astrocytes and microglia around the remaining plaques as compared with untreated animals (Figure 4) suggests this is not the mechanism of action. Astrocytes and microglia might contribute to phagocytosis of Aβ deposits,[18,19] or produce Aβ themselves and thus contribute to the deposits.[17,18] As with microglia, the role of astrocytes in the development of amyloid deposits is unclear.

There may be some concern that dissolution of existing amyloid plaques may lead to elevated levels of soluble Aβ. Several authors suggested that soluble Aβ forms mediate toxicity in AD.[20–22] There was no evidence in our study that the decrease of amyloid deposits led to any adverse effects, shortened life span or synaptic loss. The absence of any observed toxic effect of D3 in cell culture and animals, along with the drastic plaque reduction in mice and the complete inhibition of Aβ cytotoxicity in cell culture, make D3 an interesting candidate for therapeutic approaches to AD.

Even though the capability of D3 to cross the blood–brain barrier still needs to be investigated, D3 is one of the most promising candidates for therapeutic treatment and prevention of AD. In contrast to other therapeutic agents such as BACE1 and γ-secretase inhibitors, D3 is not expected to inhibit enzymes that are essential for other activities besides Aβ production, leading to adverse side effects. In addition to its potential properties as a therapeutic drug for AD treatment, D3 might also serve as a tool to study the role of Aβ plaques in AD progression.

Experimental Section

Further experimental details can be found in the Supporting Information.

Peptides: All peptides were obtained commercially (Jerini Biotools, Berlin, Germany).

Phage display screens: Biotinylated D-Aβ(1–42) was dissolved in HFIP (hexafluorisopropanol) and diluted in TBS (50 mM tris(hydroxymethyl)aminomethan (Tris)-HCl, pH 7.5, 150 mM NaCl) to a final concentration of 2 nM. This solution was transferred to a streptavidin-coated tube (Roche-Boehringer, Mannheim, Germany) and phage display was performed using the Ph.D.-12 Phage Display Peptide Library Kit (New England Biolabs, Frankfurt, Germany).

Preparation of Aβ for cell toxicity and ThT aggregation assays: L-Aβ aliquots were dissolved in an adequate volume of PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 1.8 mM KH_2PO4, pH 7.4), containing 0, 10 μM, 100 μM or 1 mM of D3, to obtain L-Aβ (1–42) concentrations of 0 μM, 10 μM and 25 μM in the respective D3 solution. All mixtures were incubated for 6 days at 37 °C.

Thioflavin T aggregation assay: 5 μL of each Aβ–peptide mixture was added to 195 μL 5 μM thioflavin T (ThT, Sigma–Aldrich, Steinheim, Germany) in 50 mM Glycin–NaOH, pH 8.5. Fluorescence was monitored immediately (t = 0 h) and after 24 h using a microplate reader at excitation and emission wavelengths of 440 nm and 490 nm, respectively (Polarstar Optima, BMG LABTECH, Jena, Germany). ThT fluorescence without addition of Aβ/peptide mixtures was subtracted from each value to correct for the fluorescence background.

Cell toxicity assays: To investigate D3 influence on cellular toxicity of L-Aβ(1–42), MTT assays were carried out in the following way: after a 6 days incubation period, 20 μL of the Aβ/D3 mixtures (see above) were added to 2×10^4 PC12 cells grown for 24 h at 37 °C in a 7.5% (*v/v*) CO_2 atmosphere. Determination of cellular 3-(4,5-dimethyl-thiazol-2-yl)2–5-diphenyl-tetrazolium bromide (MTT) reduction was carried out after 24 h incubation (37 °C in a 7.5% (*v/v*) CO_2 atmosphere) of the cells with the Aβ–D3–DMEM mixtures described above. MTT reduction was determined by measuring the difference between absorbencies at 570 nm and 630 nm. A cell viability value of 100% was defined as the MTT reduction in untreated cells. A cell viability value of 0% was defined by treatment of the PC12 cells with 0.2% Triton-X. The percentage of MTT reduction for each measurement was calculated as the fraction of the value relative to the 100% and 0% values.

In vitro disaggregation assay: To follow disaggregation of preformed fibrils, a solution of L-Aβ(1–42) in DMSO (400 μM) was diluted to 33 μM with 10 mM NaPi, pH 7.4. After 6 days incubation at 37 °C, 10 μM, ThT and 0, 1 or 3 μg μl^{-1} D3 were added, and samples with a volume of 50 μL generated. After further incubation (5, 7, 9, 11 and 13 days) at RT, ThT fluorescence was determined.

FCS measurements: Fluorescence correlation spectroscopy (FCS) measurements were performed with a Confocor I instrument (Zeiss–Evotec, Jena, Hamburg, Germany) For fluorescence detection, Aβ(1–42)-peptide (P. Henklein, Charité-Universitätsmedizin, Berlin, Germany) was labeled at the N terminus with Oregon Green (OG) (Molecular Probes, Invitrogen, Karlsruhe, Germany). A 24 well micro carrier with 20 μL sample volume (MC 384/15, Evotec Technologies, Hamburg, Germany) was used. The reaction mixture con-

tained 22 μM unlabeled Aβ(1–42) and 10 nM OG-labeled Aβ(1–42) in 10 mM sodium phosphate buffer (pH 7.2), and different concentrations of D3. OG–Aβ(1–42) were prepared from a 500 nM stock solution in 100% DMSO, stored in aliquots at −20 °C and filtered through 0.45 μm nylon filters directly prior to use. Reaction mixtures contained one molecule of OG-labeled Aβ per 2200 molecules of unlabeled Aβ. Fluorescence fluctuations in each well were recorded forty times for 30 s per sample with 500 data points resolution. Fluorescence signals with intensities of more than fivefold the mean fluctuation signal were counted as peaks. Corresponding autocorrelation functions were calculated by a hardware correlator card. Data evaluation was carried out with the Evotec software Multi-FCSaccess, version 2.05.

In vivo experiments: The experiments were conducted according with the local Institutional Animal Care and Use Committee (IACUC) guidelines. The (APPswe/PS1)E9 mice, $n = 29$; were acquired from JAX (Maine, USA) at the age of six weeks. The minipumps (Alzet, Cupertino, USA) were filled with the appropriate solution, and implanted in the brain (right dorsal hippocampus). The applied D-peptide concentration was 0.25 mg per pump, i.e., 0.25 mg in 250 μL, of the 0.25 mg peptide, 0.225 mg was unconjugated peptide, 0.025 mg was peptide conjugated with a fluorescein-5-isothiocyanate (FITC) molecule (for visualization of the peptide). After the mice were sacrificed, they were transcardially perfused at the end of the infusion period, and coronal sections were cut through the brain. Inspection of brain sections was carried out with an epifluorescent microscope. The appropriate areas (dorsal hippocampus and frontal cortex) of the brain were digitized using a Olympus DP70 digital camera, and the images were converted to grey scale using the Paint Shop Pro 7 program. The percentage of area covered by the reaction product to Aβ; was measured in the ipsi- and contralateral hippocampus (and ipsi- and contralateral frontal cortex) using the ScionImage (NIH) program. Using digital images to overlay the defined measurement area, plaques were counted in the same brain area on the adjacent sections that were stained with Congo red. Density of GFAP and CD11b staining around plaques was determined using a similar procedure.

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Keywords: neurochemistry · alzheimer's disease · therapy · peptides · phage display

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