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LPYFDa Neutralizes Amyloid- β -Induced Memory Impairment and Toxicity

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Abstract. Misfolding, oligomerization, and aggregation of the amyloid- β (A β) peptide is widely recognized as a central event in the pathogenesis of Alzheimer's disease (AD). Recent studies have identified soluble A β oligomers as the main pathogenic agents and provided evidence that such oligomeric A β aggregates are neurotoxic, disrupt synaptic plasticity, and inhibit long-term potentiation. A promising therapeutic strategy in the battle against AD is the application of short synthetic peptides which are designed to bind to specific A β -regions thereby neutralizing or interfering with the devastating properties of oligomeric A β species. In the present study, we investigated the neuroprotective properties of the amyloid sequence derived pentapeptide LPYFDa *in vitro* as well as its memory preserving capacity against A β ₄₂-induced learning deficits *in vivo*. *In vitro* we showed that neurons in culture treated with LPYFDa are protected against A β ₄₂-induced cell death. Moreover, *in vivo* LPYFDa prevented memory impairment tested in a contextual fear conditioning paradigm in mice after bilateral intrahippocampal A β ₄₂ injections. We thus showed for the first time that an anti-amyloid peptide like LPYFDa can preserve memory by reverting A β ₄₂ oligomer-induced learning deficits.

Keywords: Alzheimer's disease, amyloid- β oligomers, β -sheet breaker peptides, behavioral tests, circular dichroism spectrometry, fear conditioning, hippocampus, molecular modeling, neuroprotection, primary cortical neurons

INTRODUCTION

Progressive neurodegeneration and cognitive decline are typical features of Alzheimer's disease (AD), the most common form of dementia [1]. Besides the formation of neurofibrillary tangles, it is widely acknowledged that the aggregation of amyloid- β (A β) initiates a complex series of events that ultimately results in neuronal cell death particularly in forebrain regions, which is paralleled by the cognitive and behavioral de-

cline that is characteristic for pathogenesis of AD. A β is generated by sequential proteolytic cleavage from the amyloid- β protein precursor (A β PP) by β - and γ -secretases (amyloidogenic processing). Alternatively A β PP can be cleaved by α -secretase within the A β sequence which prevents the generation of A β peptide. Once released, A β due to its physico-chemical properties has the strong tendency to misfold, oligomerize and to aggregate into fibrils and plaques [2].

Although amyloid plaques represent a major hallmark of AD, they correlate poorly with the progression of the disease [3]. Interestingly, more recent studies have identified soluble A β -oligomer assemblies as the main pathogenic agents which, in contrast to plaques, do correlate well with the mental decline ob-

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served in AD patients [1,4–7]. Furthermore, soluble A β oligomers have been shown to be neuro- and synaptotoxic and to inhibit long-term potentiation (LTP) [8–12]. Acute application of oligomeric A β leads to internalization of glutamatergic AMPA and NMDA receptors and finally to synaptic downscaling [13,14]. However, it should be appreciated that next to its pathological properties, A β , notably in very low physiological concentrations, may have an important role in synaptic plasticity and normal brain functioning [10,15,16]. The endogenous level of A β in the brain is regulated by synaptic activity *in vivo*, suggesting a dynamic feedback loop involving A β PP metabolism and A β that may modulate synaptic activity [1]. Recently, Garcia-Osta and coworkers showed that depletion of endogenous A β by a single intrahippocampal (i.h.) administration of anti-A β -antibody leads to disrupted memory retention in rats [17]. During the pathogenesis of AD, the equilibrium of A β generation and A β clearance is disturbed, which eventually leads to elevated A β levels, increased A β aggregation, and impaired memory function [10].

Multiple therapeutic strategies have been developed since the second half of the previous century [18]. Unfortunately, most of the currently available therapies target only the symptoms, acting on presumed downstream neurotoxic pathogenic mechanisms without tackling the cause and thus hardly able to affect the progression of the disease. Experimental data from animal studies using immunotherapy in order to remove A β from the brain were highly promising [19]. However, clinical trials with active immunization against A β were halted due to severe brain inflammation and premature death of several patients [20,21].

Consequently, as a therapeutic strategy, compounds were developed that could inhibit or delay the development of A β aggregation, fibrillization, and/or plaque formation and were thus potentially capable of protecting neurons from A β toxicity. As part of this approach, small peptides derived from the A β sequence were designed, such as the pentapeptide LPYFDa, which seemed to offer a promising starting point to develop potential drugs that can somehow revert the devastating impact of A β aggregates. The advantage of such compounds, in comparison to other putative therapeutic approaches for AD such as vaccination, is that they specifically target the abnormal conformation of A β without interfering with any possible physiological function of the soluble, monomeric A β peptide.

There have been numerous attempts to develop treatments developed to interfere with various key steps in

the amyloidogenic process. Promising putative treatments may be those designed to inhibit steps that precede A β peptide aggregation, by blocking production of the toxic soluble A β oligomers in the first place, or by reversing, somehow, the toxic effect of these oligomers.

In the present study we established an *in vivo* model in the mouse for A β -induced memory impairment through a single bilateral injection of oligomeric A β_{42} into the hippocampus and explored in this model whether LPYFDa can be beneficial against the A β -induced cognitive deficits. Furthermore, we investigated the neuroprotective properties of A β -derived synthetic β -sheet breaker peptide Leu-Pro-Tyr-Phe-Asp-amide (LPYFDa) [22,24] against the neurotoxic effects of soluble A β_{42} oligomers in cultured mouse primary cortical neurons (PCN). Finally, we employed molecular modeling and docking experiments to reveal part of the putative mechanism of action of these peptides.

MATERIAL AND METHODS

Compounds

The β -sheet breaker LPYFDa was synthesized in our laboratories by a solid-phase procedure involving the use of Merrifield resin and Boc chemistry. Purity control and structure verification were carried out by amino acid analysis and mass spectrometry as previously described [25]. The control peptide (scrP), which is a scrambled version of the LPYFD peptide (Pro-Asp-Tyr-Leu-Phe-amide), and A β_{1-42} (A β_{42}) were purchased from EZBiolab Inc. (Carmel, USA). Anti-A β antibody (6E10) was obtained from Covance (Emeryville, USA). Other compounds used in this study were purchased from Invitrogen (Carlsbad, USA) or Sigma-Aldrich Corporation (St. Louis, USA).

Preparation of A β -oligomers

Oligomeric A β_{42} was prepared as described by Dahlgren and colleagues [26]. In short, the synthetic A β_{42} peptide was initially dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to a concentration of 1 mM. The peptide solution was divided into aliquots and the HFIP removed by evaporation under vacuum (SpeedVac; Savant Instruments, Hyderabad, India). The dry peptide films were stored at -20°C until further processing. Before use, the dry film A β_{42} was dissolved in anhydrous DMSO to 5 mM followed by bath sonication (Decon, Hove Sussex, UK) for 10 min,

subsequently diluted in neurobasal medium to a final concentration of 100 μ M (stock solution) and incubated at 4°C for 24 h to enable A β ₄₂ oligomerization. The aggregation state and the secondary structure of the oligomeric A β ₄₂ preparation was examined by sodium dodecyl sulfate (SDS)-PAGE Western blotting and Circular Dichroism (CD) spectrometry (Fig. 1).

Gel electrophoresis and Western blot analysis

Gel electrophoresis and Western blot analysis were adopted from Stine et al. [27]. Briefly, unheated samples in SDS sample buffer were applied to 10–20% tris-tricine gradient gels (BioRad, Munich, Germany), electrophoresed using tricine running buffer, and subsequently transferred to 0.45- μ m polyvinylidene difluoride membranes (Millipore, Bilerca, USA). Membranes were blocked with 1% I-Block (Tropix, Bedford, USA) in Trisbuffered saline containing 0.0625% Tween-20. Blots were incubated with primary antibody 6E10 overnight (1:2000; mouse monoclonal against A β residues 1–16; Covance Emeryville, USA). Immunoreactivity was detected using enhanced chemiluminescence (Pierce Biotechnology, Rockford, USA) and imaged on an Kodak X-Ray film (Eastman Kodak Company, Rochester, USA) (Fig. 1A). Molecular weight values were estimated using the PageRuler (Fermentas International, Ontario, Canada) pre-stained molecular weight marker.

CD spectrometry

CD measurements were performed at 25°C on a CD Spectrometer Model 62DS (AVIV Associates Inc., Lakewood, USA) using a quartz cell of 0.1 cm path-length. All spectra were averages of four scans; the resolution was 1 nm. The oligomerized A β was diluted in PBS to a concentration of 25 μ M. The samples were sonicated for 10 min immediately after dissolution. CD spectra were expressed as mean residue ellipticity $[\Theta]_{MR}$ in units of deg cm² dmol⁻¹ (Fig. 1B). The percentages of secondary structures were analyzed using the K2D2 program [28].

Molecular modeling

Stochastic conformational search. EDMC calculations

The conformational space was explored using the method previously employed by Liwo et al. [29], which includes the electrostatically driven Monte Carlo

(EDMC) method [30,31] implemented in the ECEPPAK [32] package. Conformational energy was evaluated using the ECEPP/3 force field [33]. Hydration energy was evaluated using a hydration-shell model with a solvent sphere radius of 1.4 Å and atomic hydration parameters that have been optimized using nonpeptide data (SRFOPT) [34,35]. In order to explore the conformational space extensively, we carried out 10 different runs, each of them with a different random number. Therefore, we collected a total of 5000 accepted conformations. Each EDMC run was terminated after 500 energy-minimized conformations had been accepted. The parameters controlling the runs were the following: a temperature of 298.15 K was used for the simulations. A temperature jump of 1,000 K was used; the maximum number of allowed repetitions of the same minimum was 50. The maximum number of electrostatically predicted conformations per iteration was 400; the maximum number of random-generated conformations per iteration was 100; the fraction of random/electrostatically predicted conformations was 0.30. The maximum number of steps at one increased temperature was 20; and the maximum number of rejected conformations until a temperature jump is executed was 100. Only *trans* peptide bonds ($\omega \cong 180^\circ$) were considered. All accepted conformations were then clustered into families using the program ANALYZE [36,37] by applying the minimal-tree clustering algorithm for separation, using all heavy atoms, energy threshold of 30 kcal.mol⁻¹, and RMSD of 0.75 Å as separation criteria. This clustering step allows a substantial reduction of the number of conformations and the elimination of repetitions. A more detailed description of the procedure used here is given in section 4.4 *Computational Methods* of reference [38].

Docking studies

Two models for A β were used as target systems; the monomeric A β ₄₂ elucidated by Crescenzi et al. (monomeric model) [39], PDB code 1IYT, and the pentameric aggregate A β ₄₂ developed by Masman et al. (pentameric model) [40]. The structures were prepared for docking study as follows: for the A β ₄₂ molecules, water molecules were removed from the PDB file and hydrogen atoms were added; Gasteiger charges, atomic solvation parameters and fragmental volumes were merged to the target system. For both LPYFDa and scrP, the structure of the most populated family (results from the EDMC calculations) was taken as initial conformation. Gasteiger charges were assigned and

Fig. 1. **A**) Oligomeric preparation of A β_{42} was examined by Western blot using the anti-A β antibody 6E10 (1:2000). Bands correspond to monomeric up to tetrameric forms of A β_{42} . **B**) CD spectrum of (x) A β_{42} in PBS after 24 hours incubation at 4°C. (—) fitted line data using K2D2 program. **C**) Schematic outline of the experimental procedure *in vitro*. PCN from C57BL/6 mouse embryos (E14) were cultured for 6 days and then treated for 24 h with different concentrations of oligomerized A β_{42} , β -sheet breaker peptides or combinations of both. On day 7 medium was completely exchanged and 24 h later the cell viability was assessed by an MTT reduction assay. **D**) Schematic outline of the experimental setup *in vivo*. C57BL/6J mice were cannulated 7 days prior i.h. injection with oligomerized A β_{42} and/or β -sheet breaker peptides. One hour after the injection the animals were trained in a contextual fear conditioning paradigm. 24 hours after the training session memory performance was assessed. Cannula position was evaluated by methylene blue injection after the behavioural test. **E**) Representative coronal brain sections of a bilateral dorsal hippocampal (i.h.) injection with methylene blue counterstained with nuclear fast red.

non-polar hydrogen atoms were merged. All torsions were allowed to rotate during docking. The docking energy grid was produced with the auxiliary program AutoGrid. The grid dimensions were 60 × 60 × 60 for the monomeric model, and 90 × 60 × 60 for the pentameric model, points along the x-, y- and z-axes, with points separated by 0.375 Å. The grids were chosen to be sufficiently large to cover significant portions of the putative binding sites. The center of the pentapeptide was positioned at the grid center. All graphic manipulations and visualizations were performed by means of the AutoDock Tools [41] and the Chimera [42] programs, and ligand docking with AUTODOCK 4 [43]. The Lamarckian genetic algorithm was utilized and the energy evaluations were set at 2.5×10^6 . A total of 250 accepted conformations were collected. Other parameters were set to default values.

Primary cortical neuron culture

PCN were prepared from embryonic brains (E14) of C57BL/6J mice. The cortices were carefully dissected, meninges were removed, and the neurons separated by trituration. Cells were plated on poly-D-lysine pre-coated plates at a density of 1.2×10^5 cells/well (96

well plates). Neurobasal medium supplemented with 2% (v/v) B27-supplement, 0.5 mM glutamine and 1% (v/v) penicillin/ streptomycin was used as a culture medium. After 48 h, neurons were treated with 10 μ M cytosine arabinoside for another 48 h to inhibit non-neuronal cell growth. Subsequently, the medium was completely exchanged, and, after 6 days of *in vitro* culture, the neurons were used for experiments.

Treatment of cells

Possible toxicity of LPYFDa, its control peptide scrP, and A β_{42} oligomers was determined by incubating neuronal cultures for 24 h with different concentrations of the peptide solutions. The neuroprotective effect of LPYFDa was assessed by incubating neurons (cultured in 96 well plates) for 24 h with 20 μ M oligomeric A β in the presence or absence of different concentrations of LPYFDa or control peptide. After the treatment, the medium was completely exchanged, and 24 h later, the cell viability was determined by an MTT-assay (Fig. 1C). All treatments were performed in triplicates and the experiments were repeated at least two times.

Determination of cell viability by MTT-assay

Neuronal viability was determined by the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described previously [44]. 1.25 mg/ml MTT solution was added to each well of a 96 well plate. After 2 h of incubation, cells were lysed in acidic propan-2-ol solution (37% HCl/ propan-2-ol: 1/166). The absorbance of each well was measured with an automated ELISA plate reader (BioRad, Munich, Germany) at 595 nm with a reference filter at 630 nm.

Animals

Behavioral experiments were performed with 9–12 weeks old male C57Bl/6J mice (Harlan, Horst, The Netherlands). Individually housed mice were maintained on a 12 h light/dark cycle (lights on at 7.00 a.m.) with food (Hopefarms, standard rodent pellets) and water *ad libitum*. A layer of sawdust served as bedding. The animals were allowed to adapt to the housing conditions for 1–2 weeks before the experiments started. The procedures concerning animal care and treatment were in accordance with the regulations of the Ethical Committee for the use of experimental animals of the University of Groningen (DEC4668C).

Animal surgery

Double guide cannulae type C235 (Plastics One, Roanoke, USA) were implanted in the brain using a Kopf stereotactic instrument during Hypnorm/Midazolam (10 ml/kg, i.p.) anesthesia under aseptic conditions as previously described [45] with anteroposterior (AP) coordinates zeroed at Bregma directed toward both dorsal hippocampi (i.h.), AP -1.5 mm, lateral 1 mm, depth 2 mm [46]. Each double guide cannula with inserted dummy cannula and dust cap was fixed to the skull with dental cement (3M ESPE AG, Seefeld, Germany). Administration of 1 mg/ml finadyne (2.5 mg/kg s.c.) before the surgery served as analgesic. The animals were allowed to recover for 6–7 days before the behavioral measurements started.

Intrahippocampal injections

Bilateral i.h. injections were performed under short isofluran anesthesia using a Hamilton microsyringe fitted to a syringe pump unit (TSE systems, Bad Homburg, Germany) at a constant rate of 0.3 μ l/min (final volume: 0.3 μ l per side). The amount of injected A β ₄₂ was of 15, 30, or 60 pmol and LPYFDa as well as the control peptide scrP in an amount of 150 pmol into the dorsal hippocampus. PBS (pH 7.5) served as vehicle. One hour after the injection the animals were subjected to a training session in a fear conditioning paradigm (Fig. 1D). The number of animals per group varied from 5 to 9.

Fear conditioning

Fear conditioning was performed in a plexiglas cage (44 × 22 × 44 cm) with constant illumination (12 V, 10W halogen lamp, 100–500 lux). The training (conditioning) consisted of a single trial. Before each individual mouse entered the box, the box was cleaned with 70% ethanol. The mouse was exposed to the conditioning context for 180 s followed by a scrambled footshock (0.7 mA, 2 s, constant current) delivered through a stainless steel grid floor. The mouse was removed from the fear conditioning box 30 s after shock termination to avoid an aversive association with the handling procedure. Memory tests were performed 24 h after fear conditioning. Contextual memory was tested in the fear conditioning box for 180 s without footshock presentation. Freezing, defined as the lack of movement except for respiration and heart beat, was assessed as the behavioral parameter of the defensive reaction of mice by a time-sampling procedure every 10 s throughout memory tests. In addition, mean activity of the animal during the training and retention test was measured with the Ethovision system (Noldus, Wageningen, The Netherlands).

Histology

Immediately after the behavioral test, mice were injected i.h. with methylene blue solution during sodium-pentobarbital anesthesia (0.1 ml/ 10 g, i.p.). Brains were removed and serially sectioned at 50 μ m. Sections were stained on glass for 5 min in 0.1% nuclear fast red solution. To identify the location of the injection, sections were analyzed using light microscopy. Only data from animals in which the proper intrahippocampal site of injection was confirmed, were evaluated (Fig. 1E).

Statistical analysis

Behavioral data were analyzed by analysis of variance (ANOVA) followed by the Bonferroni post-hoc test to determine statistical significance. For statistical analysis of the MTT assays, an unpaired Student's *t* test with unequal variance was used. A *p*-value < 0.05 was considered to be statistically significant. Data are presented as mean value $\pm$ standard error of the mean (SEM).

RESULTS

Characterization of the oligomeric A β_{42}

The state of aggregation of the oligomeric A β_{42} preparation was determined by Western blot analysis. The results confirmed that the A β_{42} preparation consisted of a mixture of small molecular weight A β_{42} oligomers from monomeric to tetrameric A β_{42} (Fig. 1A). In addition, we used CD spectrometry to characterize the secondary structure of A β_{42} in solution. We could show that our oligomeric A β_{42} preparation consists of 43.02% β -sheets, 5.40% α -helix, and 51.58% random-coil conformation (Fig. 1B), which shows a good correlation to the conformation behavior of A β_{42} aggregates observed by Masman et al. [40].

Molecular modeling and stochastic conformational search. EDMC calculations

To have a better view at the molecular level, it is crucial to assess the conformational behavior of the pentapeptides in solution. Therefore, LPYFDa and scrP were selected for energy calculations to determine the biologically relevant conformations. The results of the theoretical calculations are summarized in Table 1. Calculations yielded a large set of conformational families for each peptide studied. The total number of conformations generated was 62515 and 65056, for LPYFDa and scrP respectively, whereof 5000 conformations for each pentapeptide were accepted. In the clustering procedure, an R.M.S.D (Root Mean Square Deviation) of 0.75 Å and a ΔE of 30 kcal mol⁻¹ were used. The number of families after clustering was 220 and 323, for LPYFDa and scrP, respectively. The total number of families accepted with a relative population higher than 0.50% was 10 and 25, for LPYFDa and scrP, respectively, that sum up to *ca* 90% of all conformations. All low-energy conformers of pentapeptides studied here

Table 1

Conformational search and clustering results for LPYFDa and scrP optimized at the EDMC/SRFOPT/ECCEP/3 level of theory. Total (E_{tot} , kcal mol⁻¹) and relative (ΔE , kcal mol⁻¹) energies are also shown. All conformational families shown here have relative population (%PF) higher than 0.5%

LPYFDa	Electrostatic	Random	Thermal	Total
Generated ^a	4233	58242	40	62515
Accepted ^b	535	4431	34	5000
Family	NF ^c	%PF ^d	E_{tot}	ΔE
1	2502	50.04	-70.79	0.00
2	768	15.36	-70.76	0.03
3	370	7.40	-69.43	1.36
4	262	5.24	-70.12	0.66
5	261	5.22	-69.31	1.47
6	247	4.94	-70.25	0.54
7	62	1.24	-69.37	1.42
8	47	0.94	-70.13	0.66
9	35	0.70	-69.75	1.03
10	30	0.60	-68.45	2.34
scrP	Electrostatic	Random	Thermal	Total
Generated ^a	4288	60708	60	65056
Accepted ^b	362	4595	43	5000
Family	NF ^c	%PF ^d	E_{tot}	ΔE
1	1146	22.92	-49.71	1.18
2	635	12.70	-50.16	0.73
3	478	9.56	-49.95	0.93
4	329	6.58	-50.88	0.00
5	272	5.44	-49.66	1.22
6	250	5.00	-49.94	0.94
7	190	3.80	-50.19	0.69
8	116	2.32	-49.71	1.17
9	114	2.28	-50.57	0.31
10	101	2.02	-49.44	1.44
11	97	1.94	-49.63	1.26
12	88	1.76	-50.23	0.65
13	60	1.20	-50.25	0.64
14	53	1.06	-49.47	1.41
15	51	1.02	-49.81	1.07
16	48	0.96	-49.39	1.50
17	45	0.90	-48.58	2.30
18	39	0.78	-48.78	2.10
19	39	0.78	-48.22	2.66
20	38	0.76	-48.97	1.91
21	37	0.74	-49.23	1.65
22	32	0.64	-49.74	1.14
23	30	0.60	-48.90	1.98
24	27	0.54	-49.03	1.86
25	26	0.52	-49.07	1.81

^aNumber of conformations generated electrostatically, randomly and thermally during the conformational search.

^bNumber of conformations accepted from those generated electrostatically, randomly and thermally during the conformational search.

^cNF represents the total number of conformational families as result of the clustering run.

^d%PF represents the percent relative population based on a total of 5000 accepted conformations.

were then compared to each other. The comparison involved the spatial arrangements, relative energy, and populations. The LPYFDa evaluation showed that the

Fig. 2. Stereoview of selected conformations for LPYFDa and scrP optimized at EDMC/SRFOPT/ECCEP/3 level of theory. A) overlapping of the two most populated and energetically preferred families of LPYFDa, family 1 ($\Delta E = 0.00 \text{ kcal.mol}^{-1}$, white) and family 2 ($\Delta E = 0.03 \text{ kcal.mol}^{-1}$, pink). B) Family 4 with a relative energy (ΔE) of $0.00 \text{ kcal.mol}^{-1}$ and C) Family 1 with an $\Delta E = 1.18 \text{ kcal.mol}^{-1}$ of scrP. All hydrogen atoms have been deleted for more clarity.

most populated family (50.04%) is also the energetically preferred one, while its second most populated family (15.36%) has a relative energy of $0.03 \text{ kcal mol}^{-1}$ above the global minimum. This small energetic difference is due to a slight reorientation of the side-chain of the residue Phe (see Fig. 2A), while the rest of the structure showed approximately the same orientation. On the other hand, the most populated family of scrP (22.92%) showed a relative energy of $1.18 \text{ kcal mol}^{-1}$ above the global minimum and a relative population of 6.38%. It was observed that LPYFDa is confrontationally, generally more restricted than scrP, with a preference to form folded structures, while scrP tended to form semi-extended or fully-extended conformations. Spatial views of selected conformations, for LPYFDa and scrP are shown in Fig. 2.

Docking studies

Monomeric model

Two potential binding sites were found by using a single blind docking run (results not shown) on the monomeric A β_{42} molecule (PDB code 1IYT), which comprises two α -helix moieties (residues 8–25 and 29–

39) connected by a loop (residues 26–28). Site I encompasses the residues 21–26 containing Glu²² and Asp²³, which were previously identified as residues for aggregation of the oligomers [40,47]. Site II includes residues 6–12 located in the portion of the molecule that loses all structural organization after oligomer formation, thus forming the so-called disordered region. In Table 2 the two most populated families of the complexes of LPYFDa and scrP with the monomeric A β_{42} are summarized. LPYFDa showed lower binding energies, while site II was in general energetically preferred over site I but families on site II poorly populated. In general it can be concluded that LPYFDa binds stronger to the monomeric A β_{42} than scrP.

Pentameric model

As previously reported by Masman et al. [40], the A β_{42} aggregates contain two β -sheet moieties ($\beta 1$, residues 18–26 and $\beta 2$, residues 31–42) organized into a parallel interchain orientation, which were proposed as intermolecular binding sites for the pentameric conformation. Moreover, a third possible site for interactions with ligand molecules was postulated, which involves the $\beta 1$ and $\beta 2$ portions of the chain located at

Table 2

The two most populated families of LPYFDa and scrP found by docking simulations on the monomeric and pentameric A β_{42} peptide and the corresponding binding energies (E_B) of the complexes. The binding constant (K_B) and the relative populations (%P) are also shown

		LPYFDa				scrP			
			E_B (kcal mol ⁻¹)	K_B^a (M)	%P		E_B (kcal mol ⁻¹)	K_B^a (M)	%P
Monomeric A β_{42}	Site I	1	-4.42	5.78×10^{-4}	13.6		-3.67	2.05×10^{-3}	4.80
		2	-4.35	6.49×10^{-4}	8.00		-3.18	4.66×10^{-3}	4.00
	Site II	1	-4.95	2.35×10^{-4}	6.40		-4.10	9.89×10^{-4}	3.20
		2	-4.66	3.85×10^{-4}	3.20		-3.96	1.24×10^{-3}	2.00
Pentameric A β_{42}	TOP	1	-5.67	6.99×10^{-5}	5.60		-4.50	5.03×10^{-4}	5.20
		2	-5.04	2.02×10^{-4}	3.60		-4.73	3.39×10^{-4}	3.60
	$\beta 1$	1	-8.19	9.88×10^{-7}	8.80		-6.17	3.00×10^{-5}	5.20
		2	-7.05	6.75×10^{-6}	6.80		-5.01	2.11×10^{-4}	4.40
	$\beta 2$	1	-4.75	3.32×10^{-4}	6.00		-4.68	3.69×10^{-4}	2.80
		2	-4.77	3.20×10^{-4}	3.20		-4.92	2.48×10^{-4}	2.40

^a K_B is calculated in with the equation $K_B = \exp(\Delta G \cdot 1000 / (R \cdot T))$, where ΔG is the docking energy, Rcal is 1.98719 and TK is 298.15.

Fig. 3. A steric view of binding between the pentameric A β_{42} model and LPYFDa. Salt bridges are signaled with block-arrows. All the ligand-target contacts are depicted as wireframe spheres.

the edge of the aggregate. This site is orientated into axis of the oligomers, in the same direction where the oligomers grows by aggregation. Table 2 shows the two most populated families of the complexes of LPYFDa and ScrP with the pentameric A β_{42} . For all the sites proposed, LPYFDa showed lower binding energies than scrP. Interestingly, both pentapeptide LPYFDa and scrP, revealed an energetic preference for site

$\beta 1$. LPYFDa was designed on the basis of Soto's pentapeptide LPFFD, which derives from the amino acid sequence Leu¹⁷-Val-Phe-Phe-Ala²¹ of A β_{42} , being the $\beta 1$ portion of the aggregate. Figure 3 shows the atomic details of the interactions of the best complex (family $\beta 1$ 1, in Table 2) found between LPYFDa and the pentameric A β_{42} . It can be appreciated that the N-terminal of LPYFDa formed a double salt bridge with the Glu²²s

Fig. 4. Cell viability of PCN as determined by an MTT-assay. Neurons treated with increasing concentrations of A) A β_{42} or B) pentapeptides LPYFDa and scrP incubated for 24 hours. Neuroprotection by C) scrP or D) LPYFDa was determined by co incubating increasing concentrations of the pentapeptides with 25 μ M A β_{42} for 24 hours. Bars indicate the mean cell viability in % relative to untreated controls $\pm$ SEM. (* = significant at $p < 0.05$).

of the second and third chain of the aggregate. Also, a second salt bridge links the Lys16 to the Asp residue of LPYFDa. All salt bridges, indicated with a block-arrow that points to the positive member of the interaction, revealed an interacting distance of approximately 3.5 Å. All the ligand-target contacts are depicted as wireframe spheres. An important hydrophobic pocket was formed between the residues Phe²⁰ and Val¹⁸ of the aggregate, and the residues Leu, Pro and Phe of the pentapeptide LPYFDa. This hydrophobic pocket is indicated with a dashed line.

LPYFDa is neuroprotective against oligomeric A β_{42} in vitro

Part of our study was to assess the neuroprotective potential of the β -sheet breaker LPYFDa against A β -

induced toxicity. Therefore, we first determined the toxic effect of the A β_{42} oligomer preparation *in vitro* on cultured PCN. Neuronal cultures were exposed to increasing concentrations of oligomerized A β_{42} for 24 h, followed by an MTT-reduction assay to assess cell viability. The results showed a clear A β -induced, dose dependent decrease in cell survival reaching significance at concentrations higher than 10 μ M (Fig. 4A). For the subsequent experiments we used oligomeric A β_{42} at a concentration of 25 μ M as a toxic stimulus.

Second, we investigated if LPYFDa or the control peptide scrP exhibited any toxicity to PCN and whether the β -sheet breaker peptides were capable to overcome the toxic effect of oligomeric A β_{42} . For this purpose PCN were exposed to different concentrations of LPYFDa or scrP alone, A β_{42} alone (25 μ M) or β -sheet breaker peptides and A β_{42} together for 24 h. The results showed that LPYFDa and scrP alone were not

Fig. 5. Effect of A β ₄₂ and LPYFDa on contextual fear conditioning. A) A single i.h. injection of oligomeric A β ₄₂ led to a dose dependent significant decrease in conditioned fear when compared to a vehicle injection. B) Co-injection of A β ₄₂ with LPYFDa prevented the A β -induced memory impairment significantly, whereas the non-specific control peptide scrP failed to revert memory deficits. Bars indicate the mean relative freezing score in % $\pm$ SEM. Differences were determined by ANOVA (* = significant at $p < 0.05$).

toxic to PCN at any tested concentration (Fig. 4B). Furthermore, the control peptide scrP was not able to protect the neurons from A β -induced toxicity (Fig. 4C). In contrast, we could demonstrate a dose dependent neuroprotective effect of LPYFDa against A β -induced toxicity, reaching significance at 4 μ M LPYFDa and higher (Fig. 4D).

A single intrahippocampal injection of oligomeric A β ₄₂ induces cognitive deficits in contextual fear conditioning

One hour prior to the training session in a contextual fear conditioning paradigm C57BL/6J mice received a single injection of oligomeric A β ₄₂ (15, 30, or 60 pmol) or vehicle. Injections did not affect locomotion or the shock reaction during training (data not shown). The vehicle injected animals displayed an average relative freezing behavior of $58.9 \pm 2.9\%$, which did not differ from untreated control animals ($61.1 \pm 2.4\%$). The mice injected with A β ₄₂ showed a dose dependent decrease in freezing behavior (Fig. 5A). 15 pmol A β ₄₂ caused an average relative freezing of $50.0 \pm 4.2\%$, which was not significantly different from the vehicle-injected animals. However, the mice injected with 30 pmol and 60 pmol of A β ₄₂ had significantly reduced

freezing scores compared to the vehicle group ($36.2 \pm 4.6\%$; $p = 0.024$ and $33.3 \pm 5.3\%$; $p = 0.037$).

Next, we investigated whether LPYFDa is able to revert the A β -induced memory deficits. Therefore, we injected mice with 150 pmol LPYFDa or 150 pmol of the non specific control peptide scrP in the presence or absence of 30 pmol oligomerized A β ₄₂ into the hippocampus. The LPYFDa and the scrP injected mice showed an average freezing of $61.1 \pm 5.0\%$ and $63.0 \pm 5.5\%$ which did not significantly differ from the untreated ($61.1 \pm 2.4\%$) or vehicle injected group ($58.9 \pm 2.9\%$).

However, LPYFDa co-injected with A β ₄₂ was able to abolish the A β ₄₂ oligomer-induced memory impairment ($57.4 \pm 5.2\%$ versus $36.2 \pm 4.46\%$; $p = 0.039$). Co-injection of A β with the control peptide scrP, which resulted in an average freezing score of $48.0 \pm 4.9\%$, did not significantly reverse the A β -induced memory deficits (Fig. 5B).

These results indicate that LPYFDa can reverse the detrimental effects of A β ₄₂ oligomers and subsequent impaired memory performance.

DISCUSSION

In the present study, we could show that A β ₄₂ oligomers are toxic to primary cortical neurons in cul-

ture in a dose-dependent manner (Fig. 4A). These results are in line with studies by Dahlgren and colleagues, who reported that the oligomeric form of A β_{42} is 10 fold more toxic when compared to the fibrillar form [26]. These and our present findings support the growing general view that, in particular, oligomeric A β_{42} peptides contribute to the progressive neuronal loss and the associated memory impairment observed in AD patients. Indeed, several studies showed that elevated levels of soluble oligomeric A β correlate strongly with cognitive decline in AD patients [6,7]. Walsh and colleagues observed that a low-n oligomeric assembly of naturally secreted human A β alters hippocampal synaptic plasticity by inhibiting LTP [48]. Also the number of dendritic spines was dramatically decreased when neurons were incubated with A β oligomers, but not with monomers [48]. However, the loss in spines could be reverted by treating neurons with an anti-A β antibody [14].

A possibility to counteract the injurious effects of oligomeric A β is by modulating its aggregation. Crucial for the aggregation process of the A β molecule are the hydrophobic residues (amino acids 17–21: LVFFA) within the internal region of the A β peptide. Experiments by Hilbich and colleagues revealed that replacement of those hydrophobic residues by hydrophilic residues results in impaired fibril formation [49]. These and other findings eventually led to the concept of β -sheet breakers as therapeutic strategy for AD as proposed by Soto et al. [50,51]. The initially synthesized compounds were peptides of 11 to 5 amino acids targeting the center region of the A β peptide and evolved to compounds like LPFFD (iA β 5) and/or LPYFDa. These pentapeptides are partially homologous to this hydrophobic center region and bind with a relatively high affinity to A β [52–54] by similar intermolecular interactions, leading to a competitive replacement of A β molecules. A major drawback with peptide drugs for neurological disorders is their rapid degradation *in vivo* by proteolytic enzymes and their poor blood-brain permeability [55]. These issues were overcome by chemical modifications, like C-terminal amidation and N-terminal carboxylation, which resulted in increased half life *in vivo* and rapid brain uptake [54]. Although our knowledge of the biochemistry in respect to catabolism and brain uptake of certain aggregation inhibitor peptides has developed greatly in recent years, the detailed mechanisms of action are still poorly understood. Therefore, it is essential to elucidate and study the three dimensional structure of the A β peptide/aggregation inhibiting peptide complex to

gain more insight into the molecular dynamics of the A β aggregation process.

We used computational modeling and docking experiments to address this question. Interestingly our docking results showed that LPYFDa binds preferably to the β 1 portion of the aggregate (Table 2 and Fig. 3), which has a good correlation with the design of this pentapeptide, since LPYFDa derives from the wild-type sequence Leu¹⁷-Val-Phe-Phe-Ala²¹ of A β_{42} , which is contained in this portion of the aggregate. Our docking results did not show any binding preference of LPYFDa (nor scrP) for the monomeric or the pentameric A β .

In the present study, we showed that the pentapeptide LPYFDa can protect cultured neurons from oligomeric A β -induced cell death. Datki and colleagues reported similar results on neuronal-like cell lines, e.g., SHSY-5Y cells. They could show that a 5-fold molar excess of LPYFDa protects these cells from toxic effects of fibrillar A β [24,56]. In our study we could confirm that a molar excess of LPYFDa can prevent A β_{42} mediated neurotoxicity. Moreover, we demonstrated a dose-dependent protective effect of LPYFDa and that this pentapeptide already has significant neuroprotective properties even with a 6-fold molar excess of oligomeric A β_{42} . Our results are also in agreement with other studies reporting protection by LPYFDa against the rapid neuromodulatory action of fibrillar A β_{42} demonstrated by *in vitro* and *in vivo* electrophysiology [23].

We found that a single bilateral intrahippocampal injection of oligomeric A β_{42} impairs memory formation if applied 1 h before the training session in a contextual fear conditioning paradigm. These findings are in line with several other studies, which consistently report on memory deficits after intracerebral injections of A β_{42} peptides, although the experimental conditions vary in terms of the injected peptide, injection procedure, and behavioral tasks employed [57–62]. It should be noted that these injections obviously lead to A β levels beyond the basal levels necessary for proper synaptic functioning. Garcia-Osta showed that neutralizing endogenous A β by an anti-A β antibody resulted in memory impairment which implies that physiological soluble A β levels are required for proper memory function [17], which was recently confirmed by a study of Morley et al. [63].

The peptide LPFFD, which was designed by the Soto group, was able to prevent fibrillogenesis in a rat brain model [64] and to reduce A β deposition in a transgenic AD mouse model [54]. Most interestingly, iA β 5p was shown to reverse the memory impairment caused by

intrahippocampal injections of A β fibrils in rats [65]. However, these studies did not reveal whether impairment of learning and memory after oligomeric A β injections could be counteracted by such compounds. A novelty of our study is that we used A β oligomers instead of the less effective A β fibrils. Therefore, an important aim of our study was to establish if a compound like LPYFDa is able to prevent A β oligomer-induced learning and memory deficits. We showed that a 5-fold molar excess of LPYFDa to A β could overcome the detrimental effects of A β oligomers on memory. Thus, we provide evidence that so-called β -sheet breaker peptides such as LPYFDa bear therapeutic potential against A β -induced memory impairment. Moreover, it was recently demonstrated that intraperitoneally administered LPYFDa is able to cross the blood brain barrier and protects synapses against excitatory action of fibrillar A β [22].

The mechanism of how these pentapeptides exert their protective effects on cell death and behavior is not yet fully understood. However, it should be noted that the two actions of A β reported here, namely, the neuronal degeneration following application of A β oligomers *in vitro* and memory loss following injections of A β oligomers *in vivo* may be unrelated. In hippocampal tissue extracted one hour after A β injection, we were unable to detect any caspase-3 cleavage which would indicate apoptotic cell death (data not shown). Furthermore, there are no studies so far that show toxic effects of oligomeric A β on neurons *in vivo*. Therefore, the effects on memory following an hour post-injection may not have a direct correlation with neuronal degeneration, but could simply reflect the effects of A β on synaptic integrity. It is well documented that the main effects of A β on synapses are inhibition of LTP [9,66] and elimination of postsynaptic glutamate receptors [8, 67,68]. Remarkably, the A β -induced synaptic dysfunction occurs rather rapidly, starting 20 minutes after application, and does not need chronic exposure [69–72]. Furthermore, it is likely that the injected oligomeric A β does not remain unchanged in terms of its conformation, and the effects on memory may be due to changes of A β to other conformations. The importance of the A β conformation on memory was demonstrated by Lesne and collaborators [73], who showed that only specific A β protein assemblies in the brain are able to impair memory [73].

It remains elusive whether aggregation inhibiting peptides like LPYFDa directly bind to A β , and thereby prevent possible interactions between A β and neuronal membrane proteins, and in this way neutralize its toxic

effect. We could consider two options of interaction with A β : 1) the pentapeptides bind to the monomeric A β , thus preventing and/or retarding the formation of toxic oligomers; and 2) the pentapeptides bind the A β oligomers already formed preventing and/or modulating, somehow, its neurotoxic properties. On the other hand, both above mentioned possibilities might act simultaneously comprising a third possible way of action of this peptide toward its neuroprotective effects.

Being aware of the many possible biological pathways that these peptides might follow while inducing neuroprotection, it is interesting to see whether these peptides show a preference to bind the monomeric A β or its soluble oligomers. Previous studies using CD spectrometry and molecular docking studies have been carried out on LPYFDa and other so-called β -sheet breaker peptides [24,52,74,75] but none of them so far have demonstrated proof at the molecular level of the β -sheet breaking properties of these peptides. We also might consider the possibility that these peptides might act as “glue” that promote elongation to biologically inert larger aggregates, or conversely, bind to the monomeric A β and this way inhibits oligomer formation. By either of these actions, or both, the neurotoxicity of A β is decreased or reverted.

In summary, our findings provide evidence on how and where LPYFD interacts with A β mono- and oligomers, that LPYFDa neutralizes the neurotoxic activity of soluble A β oligomers, and in the present conditions can effectively prevent the oligomer-induced deficits in memory performance.

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