

Globular and Protofibrillar A β Aggregates Impair Neurotransmission by Different Mechanisms

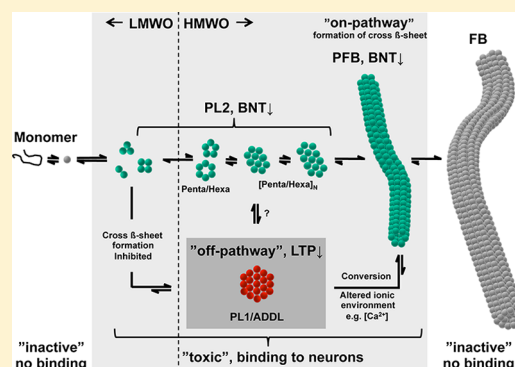
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S Supporting Information

ABSTRACT: In Alzheimer's disease, substantial evidence indicates the causative role of soluble amyloid β (A β) aggregates. Although a variety of A β assemblies have been described, the debate about their individual relevance is still ongoing. One critical issue hampering this debate is the use of different methods for the characterization of endogenous and synthetic peptide and their intrinsic limitations for distinguishing A β aggregates. Here, we used different protocols for the establishment of prefibrillar A β assemblies with varying morphologies and sizes and compared them in a head-to-head fashion. Aggregation was characterized via the monomeric peptide over time until spheroidal, protofibrillar, or fibrillar A β aggregates were predominant. It could be shown that a change in the ionic environment induced a structural rearrangement, which consequently confounds the delineation of a measured neurotoxicity toward a distinct A β assembly. Here, neuronal binding and hippocampal neurotransmission were found to be suitable to account for the synaptotoxicity to different A β assemblies, based on the stability of the applied A β aggregates in these settings. In contrast to monomeric or fibrillar A β , different prefibrillar A β aggregates targeted neurons and impaired hippocampal neurotransmission with nanomolar potency, albeit by different modalities. Spheroidal A β aggregates inhibited NMDAR-dependent long-term potentiation, as opposed to protofibrillar A β aggregates, which inhibited AMPAR-dominated basal neurotransmission. In addition, a provoked structural conversion of spheroidal to protofibrillar A β assemblies resulted in a time-dependent suppression of basal neurotransmission, indicative of a mechanistic switch in synaptic impairment. Thus, we emphasize the importance of addressing the metastability of *prefacto* characterized A β aggregates in assigning a biological effect.



Despite the well-accepted pathogenic role of amyloid β (A β) in Alzheimer's disease,¹ the underlying pathogenic mechanism is still elusive.² A β is generated from amyloid precursor protein by sequential proteolysis.³ This is followed by its self-association from monomeric to soluble prefibrillar A β , which further assembles into insoluble A β fibrils that deposit in the brain as amyloid plaques. Because the number of these plaques, as opposed to the presence of soluble A β -aggregates,⁴ does not correlate well with the severity of dementia,⁵ the amyloid hypothesis has been reformulated, positioning soluble A β aggregates as the prime toxic agent, causing synapse loss and decreased cognitive performance.⁶ Substantial efforts have been made to identify the "most" toxic A β aggregate, derived from synthetic peptide and endogenous sources, i.e., cell culture, transgenic animals, and post mortem tissue. These studies resulted in the description of different soluble, prefibrillar A β species, which all show pathophysiological relevance, i.e., neurotoxicity and impaired neurotransmission.⁷

Although the parameters used predominantly for the differentiation of A β aggregates are size and molecular weight

(MW), a direct comparison of these aggregates is difficult because this comparison is based on different methods.⁷ Furthermore, some of these methods affect aggregation per se, e.g., the presence of SDS,^{8,9} limiting the suitability of SDS-PAGE for characterization. Other methods such as atomic force microscopy (AFM) and transmission electron microscopy (TEM) are restricted to high protein concentrations and purities; however, these do offer structural information on a nanometer scale.^{10,11}

Because of the difficulty in purifying endogenous A β species without interfering with the native state of the assemblies,¹² synthetic A β has been favored for biophysical characterization, using various A β peptides, as well as a range of solubilization and aggregation conditions^{10,13–15} to generate A β aggregates of different sizes and morphologies.⁷ Most of these A β species are temporally transient and metastable intermediates "on-path-

Received: December 9, 2012

Revised: January 28, 2013

Published: February 1, 2013



way" to fibrils (e.g., hexamer and protofibrils), but some [e.g., A β oligomers (A β O) or "Alzheimer-derived diffusible ligands" (ADDLs)] reach equilibrium, i.e., do not undergo a significant change over the time of observation in the respective aggregation buffer. These species are considered as "off-pathway" assemblies, indicative of a nonlinear process of aggregation.^{16,17} In addition, several studies showed that a change in the molecular interactions induced by an altered environment (e.g., buffer, peptide concentration, and temperature) results in a rapid rearrangement of *prefacto*-characterized A β aggregates.^{18,19} Thus, the metastability hampers the elucidation of aggregate structure and also the characterization of pathophysiological effects. Consequently, the stability of *prefacto*-characterized A β aggregates in the experimental paradigms should be evaluated cautiously before a pathogenic mechanism is attributed to a certain A β species. Although different soluble A β species have been proposed to mediate synaptotoxicity via different mechanisms and targets,^{7,17,18,20,21} studies aiming for a head-to-head comparison of different prefibrillar A β aggregates are rarely found. Here, the morphology and MW of monomeric, spheroidal, protofibrillar, and fibrillar A β assemblies were characterized by AFM and dynamic (DLS) and static multiangle (MALS) light scattering, while minimizing interference on A β aggregation. Furthermore, pathophysiological effects were assessed under conditions where A β aggregate reorganization was minimized. Herein, neuronal interaction was found only for prefibrillar A β assemblies mediating impairment of neurotransmission by at least two independent pathways.

EXPERIMENTAL PROCEDURES

A β Preparations. A β 40 or A β 42 (desalted peptide, Bachem) was dissolved in HFIP (Fluka) to a final concentration of 1 mM, sonified for 10 min, gently shaken for 1 h at room temperature, snap-frozen in liquid nitrogen, and lyophilized. The peptide was stored at -80°C until it was used. If not stated otherwise, A β was solubilized at 1 mM in NaOH (10 mM), sonified for 2 min, and diluted to a final concentration of 100 μM in the respective aggregation buffer. After incubation, all preparations except fibrils were centrifuged (15000g for 10 min), and the supernatants were analyzed. For monomeric A β (Mono), solubilized A β 40 was diluted in PBS, centrifuged, and used within 1 h. For prefibrillar A β aggregates (PL1 and PL2), solubilized A β 42 was diluted in phenol red-free Ham's F12 (PL1; Invitrogen) or DMEM (PL2; Invitrogen) and incubated at 4°C for 20 h. For protofibrils (PFB), solubilized A β 42 was diluted in PBS and incubated at 4°C for 20 h. For fibrils (FB), solubilized A β 42 was diluted in 10 mM HCl and incubated at 37°C for 20 h.

DLS. Cumulative fitting was used to measure the global hydrodynamic radius ($R_{H,c}$) of the aggregate solution, until pseudoequilibrium was reached. The regularization method was used to determine the size distribution of A β aggregates ($R_{H,r}$).²²

AF4-MALS. AF4 separates molecules by differences in Brownian motion, without a stationary phase, minimizing shear forces and protein–matrix interactions.^{23,24} The AF4 system (Eclipse III+, Wyatt Technology) was controlled by a high-performance liquid chromatography system (Agilent, 1200 series), defining sample loading (10–50 μg /injection) and flow conditions. AF4 was optimized to separate particles with a molecular weight of 4–2000 kDa using a 1 kDa cutoff PES membrane, a 480 μm spacer, PBS as the eluent, and the

following flow conditions: detector flow, 1 mL/min; cross-flow, 3 mL/min from 0 to 10 min, 3.0 to 0.1 mL/min from 10 to 15 min, and 0.1 mL/min from 15 to 30 min. Fractions were analyzed by UV₂₂₀/UV₂₈₀ and MALS (DAWN Heleos, Wyatt Technology). Data from MALS were analyzed by ASTRA (Wyatt Technology).

AFM. Measurements were performed with a NanoScope IIIa (Veeco) in tapping mode, using RTESP-SS tips (Veeco) and muscovite Mica. The scanning area was set to $2\ \mu\text{m} \times 2\ \mu\text{m}$ at $\leq 2\ \text{Hz}$. Captured data were processed with SPIP (Image Metrology). Thirty microliters of the A β preparation (10 μM) was added for 2 min at room temperature, rinsed with 2 mL of Milli-Q-water, and dried immediately with nitrogen for 2 min. The spatial tilt of raw images was fit by a polynomial fit (third-order) and line-wise correction. Particle recognition using the watershed algorithm²⁵ was validated with standardized gold particles 2, 5.5, and 14.5 nm in diameter (Ted Pella Inc.). Lateral dimensions were used as relative measures only according to tip size broadening. The watershed algorithm (optimized for spheroidal structures) was not suitable for particle recognition of fibrils, because of oversegmentation (i.e., underestimation of length). Therefore, FB was analyzed using the threshold algorithm.

Primary Neuronal Culture. Procedures involving animals (Janvier, Le Genest Saint Isle, France) and their care were conducted in a manner consistent with institutional and European Union guidelines (EEC Council Directive 86/609) and were approved by the Ethical Committee of the respective regional councils. Hippocampal neurons derived from CS7BL6 mice were prepared as previously described by Brewer et al.²⁶

Neurotoxicity. Neuronal cultures (DIV21) were treated with A β aggregates (3 μM) prediluted in neurobasal medium and incubated for 48 h. The viability of cells was assessed by MTT assay according to the manual (Invitrogen).

Binding of neuronal A β to matured hippocampal neurons (DIV21) was assessed by a modified protocol of Lacor et al.,²⁷ using biotinylated A β aggregates, detected by streptavidin-AF488 (1 μg /mL; Invitrogen). Neurons were stained with anti-CaMK (1 μg /mL; Enzo Life Science) using the IR-700 anti-mouse secondary antibody (0.1 μg /mL; Licor Odyssey).

Electrophysiology. Male Wistar rats (7 weeks old) were sacrificed by decapitation. The preparation of brain slices and recording of LTP were conducted according to the method of Kroker et al.²⁸ Briefly, transverse hippocampal slices (400 μm) were prepared and allowed to recover at room temperature in aCSF. After equilibration for at least 30 min, field excitatory postsynaptic potentials (fEPSPs) were elicited in the CA1 region by stimulation of the Schaffer collateral-commissural fibers in the *stratum radiatum*. The amplitudes of fEPSPs were used as the parameter of interest.²⁹ NMDA receptor (NMDAR)-dependent LTP was induced by high-frequency stimulation (HFS; 100 Hz, 1 s²⁸).

Presentation of Data and Statistical Analysis. All results were reproduced at least three times in independent experiments. If not stated otherwise, data represent mean values \pm the standard deviation of n experiments. Comparison of groups was analyzed by an unpaired t test or one-way ANOVA followed by Bonferroni post hoc test, using Prism (GraphPad version 5.04). $p < 0.05$ was considered statistically significant. The significance level is indicated as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. n.s. indicates nonsignificant differences.

Further methodological details are given in the Supporting Information.

RESULTS

Characterization of Different A β Assemblies. The initial solubilization of the A β peptide is a crucial step in establishing robust and reproducible A β preparations. Therefore, we compared different solvents for solubilization and analyzed the morphology and size of the resulting A β aggregates. We confirmed that solubilization in hexafluoro-2-propanol (HFIP) and NaOH was superior to that in DMSO for the initiation of A β aggregation from the monomeric state, based on the generation of monodisperse A β solutions as assessed by DLS. A β solubilized in DMSO revealed polydisperse solutions with an R_H of >3 nm. A β 42 in NaOH or HFIP showed a hydrodynamic radius (R_H) of 1.8 nm, which increased with the time of incubation in contrast to A β 40 showing a stable R_H of monomeric A β [1.1–1.3 nm (Figure S2C of the Supporting Information and Table 1A)].

The subsequent dilution of NaOH-solubilized A β in different buffers induced aggregation and resulted in pseudostable aggregates (Figure S2D of the Supporting Information) with

Table 1

Solubilization of A β in Different Solvents ^a				
protein	MW (kDa)	$R_{H,r}$ (nm)	$R_{H,calc}$ (nm)	n_{exp}
aprotinin	6.5	1.7 \pm 0.2	1.6	5
BSA	66	3.3 \pm 0.2	3.5	5
thyroglobulin	669	10.1 \pm 0.3	10.1	5
A β 40-HFIP	4.5	1.5 \pm 0.4	1.3	3
A β 42-HFIP	4.5	1.3 \pm 0.6	1.3	3
A β 40-NaOH	4.5	1.5 \pm 0.4	1.3	3
A β 42-NaOH	4.5	1.8 \pm 0.4	1.3	3
A β 40-DMSO				
P1 (35%)	4.5	2–10	1.3	3
P2 (65%)	4.5	>150	1.3	3
A β 42-DMSO				
P1 (40%)	4.5	2–10	1.3	3
P2 (60%)	4.5	>500	1.3	3
Particle Analysis of A β Preparations in AFM ^b				
A β preparation	height (nm)	length (nm)	N	n_{exp}
CTL (no A β)	0.2 \pm 0.1	71.3 \pm 57.6	1186	5
Mono	1.1 \pm 0.4	11.0 \pm 6.6	5789	10
PL1	6.4 \pm 3.1	24.1 \pm 6.9	5162	10
PL2 Average	2.4 \pm 2.21	20.3 \pm 15.1	2168	5
P1	1.5 \pm 0.6	20.5 \pm 14.8	1627	5
P2	5.6 \pm 1.7	37.6 \pm 16.3	541	5
PFB	5.3 \pm 1.0	78.1 \pm 61.8	5053	10
FB	6.2 \pm 1.5	211.8 \pm 335.2	710	10

^aMeasured $R_{H,r}$ (DLS) and calculated R_H ($R_{H,calc}$) of standard proteins (0.5 mg/mL in PBS) and A β peptides (0.5 mg/mL) in different solvents. $R_{H,calc}$ was calculated on the basis of MW with Dynamics (Wyatt Technology). Measured and calculated R_H values for standard proteins differed by <10%. For A β solubilization in NaOH or HFIP, $R_{H,r}$ did not differ significantly from $R_{H,calc}$. However, A β solubilized in DMSO revealed two populations (P1 and P2); in this instance, averaged values are shown. ^bData represent means \pm the standard deviation of N particles, derived from n independent experiments; all prefibrillar preparations as well as Mono and blank mica differed significantly from each other in height and length (***p < 0.001), with the exception of PL1 and PL2 (no significant difference in length). PL2 is depicted as average and as populations 1 and 2 (P1 and P2, respectively) based on the observed bimodal distribution. FB was not included in this statistical analysis because of the different particle recognition algorithm.

different morphology and size distributions (Figure 1), which we define as monomeric (Mono), oligomeric (PL1 and PL2), protofibrillar (PFB), and fibrillar (FB) preparations. A β 42 instantaneously aggregated in all buffers that were investigated, thus precluding its use as a monomeric preparation. In contrast, A β 40 revealed a constant R_H in line with monomeric A β [Mono (Figure S2D of the Supporting Information)]. The established preparations revealed conformational differences (Figure S5 of the Supporting Information). ThioT fluorescence did not increase for Mono and PL1 but did significantly for PFB and FB, indicating the generation of cross β -sheet, which was confirmed by the appearance of curvilinear structures in TEM. A11 dot blots of PL1 and PL2 resulted in strong A11 immunoreactivity, and Mono, PFB, and FB are A11-negative.

Further characterization of the individual A β preparations was performed by AFM followed by semiautomated particle recognition, upon validation with standardized gold colloids (Figure S1 of the Supporting Information) to assess the morphology (height and length) of the formed A β aggregates. The MW distribution of these preparations was determined by asymmetrical flow field-flow fractionation (AF4) followed by MALS (Figure 1 and Table 1B). For the determination of MW, we considered AF4-MALS superior to classical size-exclusion chromatography and electrophoresis based on a stable ionic environment and fast separation without the need of a stationary matrix. The combination of AFM, DLS, and AF4-MALS allowed a precise characterization of the morphology, size, and MW with minimized interference with the delicate equilibrium of the A β aggregate distributions in the respective preparations. Herein, Mono appeared as unstructured particles with a monodisperse height of 1.1 \pm 0.4 nm and a corresponding MW of 4.5 kDa at peak (85%). PL1 appeared as spheroidal particles with a monodisperse height distribution of 6.4 \pm 3.1 nm with <20 kDa aggregates (23%), the predominant peak at 73 kDa (46%), and the remaining 21% larger than 100 kDa. PL2 exhibited a height of 2.4 \pm 2.1 nm; however, heterogeneity was indicated by two maxima (P1 and P2) at 1.5 nm (PL2-P1, 75%) and 5.6 nm (PL2-P2, 25%), with the latter being elongated (40 nm) compared to PL1 and PL2-P1 (both 20 nm). In AF4-MALS, PL2 showed peaks at 6 kDa (33%) and 60 kDa (42%), with 25% larger than 100 kDa. PFB showed elongated particles (78 nm) with a height of 5.3 \pm 1.0 nm, comparable to PL1 and PL2-P2, and a trimodal distribution in AF4-MALS, with maxima at 6 kDa (10%), a shoulder at 60 kDa (23%), and a second peak at 644 kDa (67%). FB exhibited fibrillar assemblies with a height of \sim 6 nm and a length of >1 μ m obtained by manual segmentation. The particle recognition analysis in AFM and MW determination by AF4-MALS was not suitable because of oversegmentation (Figure S3 of the Supporting Information) and inversed elution, respectively.²⁴ In summary, our setup revealed continuous MW distributions for all A β preparations and no single molecular entity. These preparations were stable in their individual environment and varied significantly from each other with regard to conformation, predominant size, morphology, and MW.

A β Aggregates Are Metastable. Because ab initio A β aggregation can be affected by the selected ionic environment,¹⁰ we characterized the stability in DLS kinetics of prefabricated A β aggregates under the conditions applied to assess their bioactivity, i.e., neurotoxicity (Figure 2), neuronal binding, and neurotransmission (Figure 3 and Figure S6 of the Supporting Information). In DLS, the R_H has been analyzed by

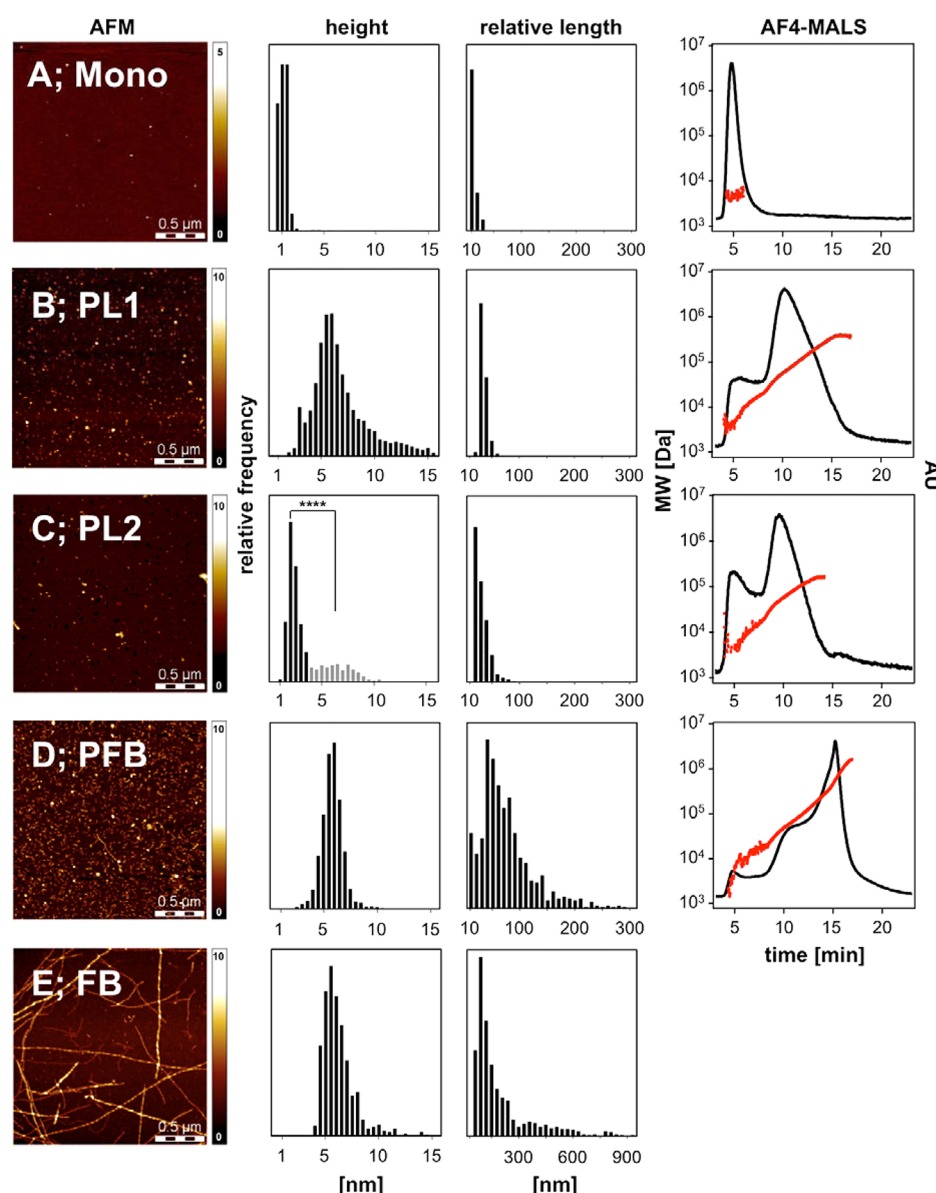


Figure 1. Distribution of height, apparent length, and MW determined by AFM-SPIP and AF4-MALS for A β monomer (A), PL1 (B), PL2 (C), PFB (D), and FB (E). Via AFM, PL2 revealed a bimodal height distribution, which was further distinguished in PL2-P1 (<3 nm) and PL2-P2 (>3 nm). P1 and P2 differed significantly in height and length ($p < 0.0001$). Data represent cumulative distributions of at least five individual experiments for each preparation. AFM images have dimensions of 2 $\mu\text{m} \times 2 \mu\text{m}$. The obtained height and relative length are listed in Table 1. Via AF4-MALS, the mass distribution (black) and MW (red) of A β preparations were obtained.

a cumulative ($R_{H,c}$) and a regularized fit ($R_{H,r}$) to determine a global change in size and the size distribution of A β aggregates, respectively. Centrifugation and dilution in the respective aggregation buffer (binding experiments) did not change $R_{H,c}$ or $R_{H,r}$ as exemplified for PL1 6 h postdilution (Figure 2A). However, dilution of PL1 in artificial cerebrospinal fluid (aCSF, neurotransmission experiments) induced aggregate reorganization, shown by an increase in $R_{H,c}$ and the occurrence of large aggregates after 1 h [PL1_{aged} (Figure 2B)] and the appearance of protofibrillar and fibrillar aggregates in AFM (Figure 2D,E). Similarly, dilution of Mono in NB medium (neurotoxicity experiments) increased $R_{H,c}$, indicative of the generation of large aggregates, confirmed by 40% insoluble A β recovered in the pellet after 48 h (Figure 2G). A critical factor, sufficient to induce reorganization of preformed A β aggregates, was the Ca²⁺ concentration, which was low in the buffers used for

Mono and PL1 (0 and 0.3 mM, respectively) but high in NB medium (1.8 mM) and aCSF (2 mM). Dilution of PL1 in high-Ca²⁺ concentration Ham's F12 (2 mM) was sufficient to induce reorganization of PL1 (Figure 2C) as it was observed for dilution of PL1 in aCSF. In summary, subtle changes in the ionic environment were sufficient to induce rapid reorganization of A β aggregates.

Bioactivity of Different A β Aggregates. MTT toxicity, differential neuronal binding, and hippocampal neurotransmission have been applied to test whether different A β aggregates also reveal different neuronal impairment or toxicity. Modest but significant neurotoxicity was observed for all A β preparations when compared to buffer controls, but not between individual A β preparations (Figure 2F). The lack of aggregate stability over 48 h underlines the limitations of the MTT toxicity experiment for attributing a neurotoxic effect to a

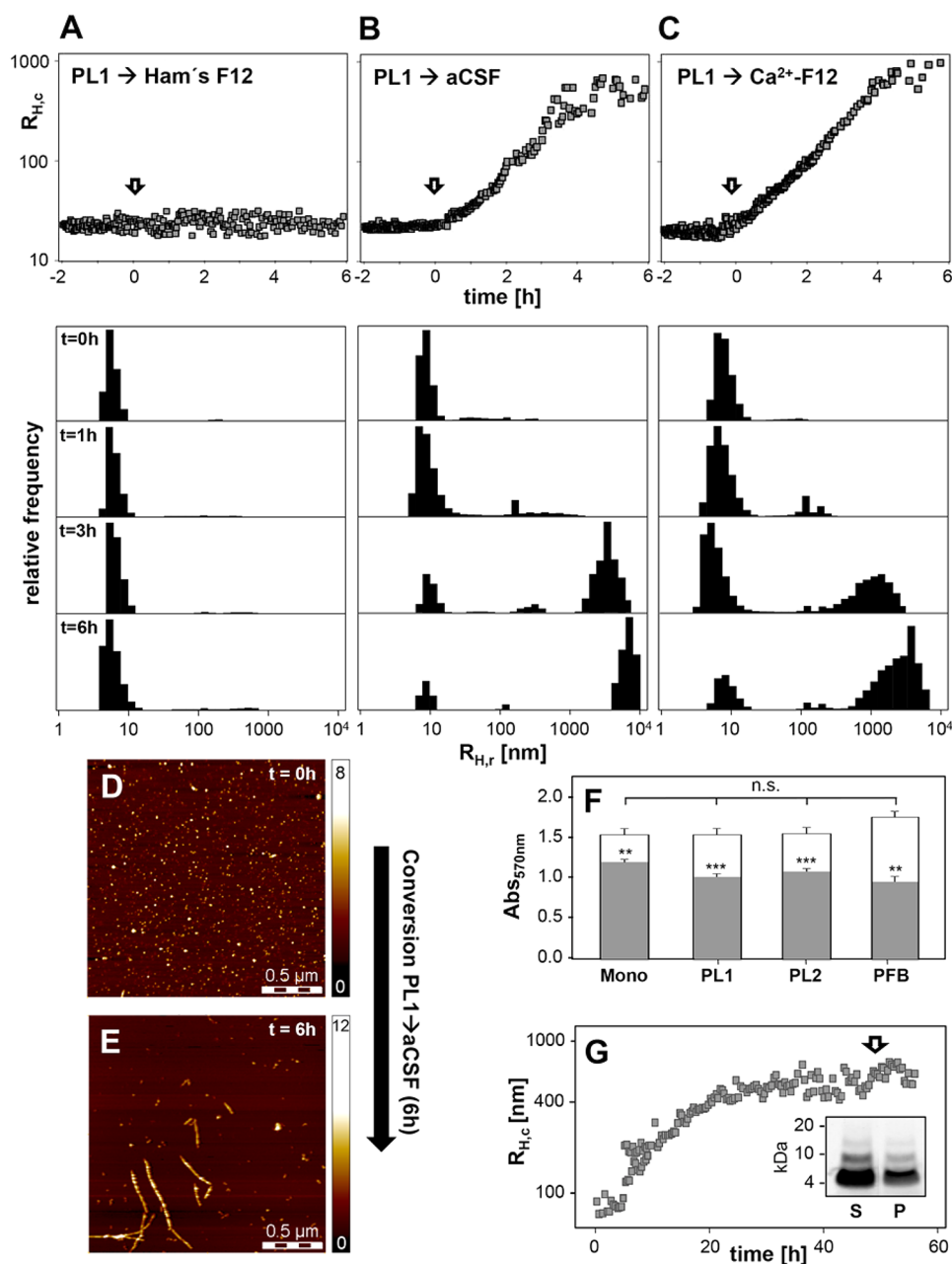


Figure 2. Metastability of Aβ aggregates. Dilution of PL1 in aCSF (1:3) and incubation for 6 h (room temperature) resulted in conversion to protofibrillar and fibrillar aggregates as shown by AFM (D and E). (A–C) Stability of PL1 after centrifugation ($t = -2$ h) and dilution ($t = 0$ h) in Ham's F12 (A), aCSF (B), and high-Ca²⁺ concentration F12 (C). PL1 (100 μM) was centrifuged (10 min at 20000g) and measured in DLS for 2 h (-2 to 0 h), followed by dilution in the respective medium (1:3, arrow), and measured for an additional 6 h. $R_{H,r}$ distributions (bottom panels of A–C) were determined at the indicated time points. Dilution of OL2 in aCSF or high-Ca²⁺ concentration F12 caused a time-dependent increase in $R_{H,r}$, whereas dilution in Ham's F12 did not induce a change in $R_{H,r}$. Large aggregates ($R_H > 100$ nm) became predominant after 3 h in panels B and C. (F) Neurotoxicity of Aβ aggregates on primary hippocampal neurons revealed modest neurotoxicity of Aβ (3 μM; 55–78% viability, *** $p < 0.001$) compared to aggregation buffer without Aβ (white bars). (G) DLS kinetics of Mono upon dilution in NB medium. The inset shows centrifugation of the diluted monomeric preparation after 48 h (arrow; 15000g for 10 min) followed by 6E10-WB indicated for insoluble aggregates (S, supernatant; P, pellet).

certain Aβ species. For binding experiments, Aβ was incubated for not more than 1 h in the respective aggregation buffer (instead of dilution in the neuronal culture medium) on neurons to minimize reorganization. Here, N-terminal biotinylation affected neither the morphology nor the size of Aβ aggregates, as exemplified by PL1 (Figure S6A of the Supporting Information). Binding was restricted to prefibrillar aggregates and mature neurons (DIV21). PL1, PL2, and PFB

bound dose-dependently (Figure S6 of the Supporting Information) in a punctate pattern in contrast to Mono or FB (Figure 3). Furthermore, binding was not observed in young neurons [DIV7 (Figure S6E of the Supporting Information)].

Impairment of hippocampal neurotransmission was assessed by brief Aβ incubations in aCSF within 30 min before reorganization was observed (Figure 2F). Field excitatory

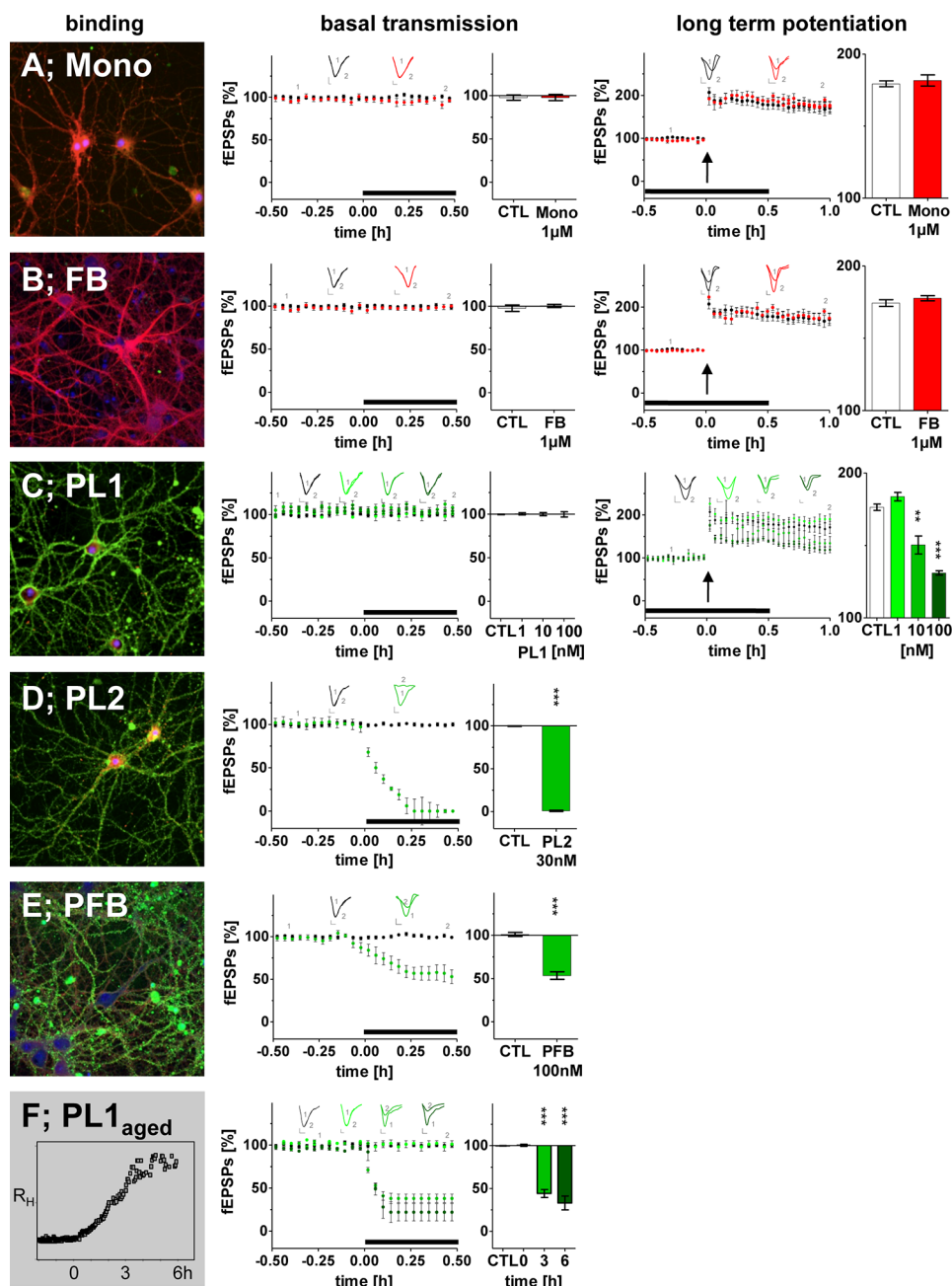


Figure 3. Soluble $A\beta$ aggregates differentially bound to mature hippocampal neurons and affected neurotransmission by different mechanisms. Mono (A) and FB (B) showed no binding, in contrast to the punctate binding of the prefibrillar preparations [biotinyl- $A\beta$ stained with Streptavidin-AF488 (green)]. AMPAR-dependent fEPSPs were recorded in the CA1 region of hippocampal rat slices for 30 min, followed by administration of freshly diluted $A\beta$ preparations. If basal transmission was not affected by the respective $A\beta$ assemblies, LTP was induced by HFS (100 Hz, 1 s). Individual fEPSPs are shown before (1) and after (2) $A\beta$ application (horizontal bar). Mono and FB had no effect on basal transmission or LTP (A and B, 1 μ M). PL1 had no effect on synaptic transmission up to 100 nM. (C) LTP was reduced by PL1 dose-dependently, with the maximal effect occurring at 100 nM. (D) PL2 completely inhibited synaptic transmission at 30 nM. (E) PFB significantly inhibited synaptic transmission at 100 nM. (F) PL1 aged in aCSF caused an inhibition of basal transmission depending on the preincubation time in aCSF [10 nM (E)]. The fEPSP amplitudes are given in percent of baseline (mean \pm standard error of the mean of at least five independent experiments).

postsynaptic potentials (fEPSPs) were analyzed before [basal neurotransmission (BNT)] and after induction of long-term potentiation (LTP) by high-frequency stimuli (100 Hz, 1 s) in the presence of $A\beta$ (Figure 3). To avoid buffer-related artifacts, all $A\beta$ preparations were compared to control measurements in the respective $A\beta$ -free aggregation buffers (%CTL). FB and Mono (both up to 1 μ M) affected neither BNT nor LTP (Figure 3A,B). PL1 did not affect BNT at 100 nM (CTL, 100 \pm 1; PL1, 100 \pm 1, n.s.); however, it did inhibit LTP dose-

dependently (Figure 3C; CTL, 176 \pm 2; PL1, 184 \pm 3 at 1 nM, 150 \pm 6** at 10 nM, and 131 \pm 2*** at 100 nM). In contrast, PL2 (30 nM) inhibited BNT completely within 15 min (Figure 3D; CTL, 100 \pm 1; PL2, 0 \pm 0***). Thus, induction of LTP could not be addressed. Similarly, PFB significantly inhibited BNT at 100 nM (Figure 3E; CTL, 100 \pm 1; PFB, 56 \pm 2***). Remarkably, preincubation of PL1 in aCSF for 3 or 6 h prior to the experiment (PL1_{aged}) led to an inhibition of BNT, which increased with time of aging (Figure 3F; 100 \pm 1 at 0 h, 44 \pm

5*** at 3 h, and $33 \pm 8^{***}$ at 6 h). Thus, the conversion of spheroidal PL1 to protofibrillar/fibrillar PL1_{aged} was paralleled by a switch from impaired LTP to BNT. In summary, neither monomeric nor fibrillar A β bound to neurons or affected neurotransmission. In contrast, the prefibrillar A β preparations bound to neurons and affected two different modalities of hippocampal neurotransmission, i.e., either BNT (PL2, PFB, and PL1_{aged}) or LTP (PL1).

DISCUSSION

Soluble A β aggregates are considered to cause synaptotoxicity and cognitive decline in AD.^{6,30} In the past two decades, cell-derived and synthetic A β species have been extensively characterized. This has led to the description of a variety of A β species claiming distinct entities and pathogenic modalities, resulting in a controversial debate about the pathophysiological relevance of a distinct A β aggregate in AD.⁷

The methods generally applied for characterization of endogenous or synthetic A β do not resolve this debate. Nevertheless, the accessible methods allow for comparison of synthetic spheroidal and protofibrillar A β aggregates and their associated bioactivity, which has not been investigated intensively to the best of our knowledge. Here, synthetic A β preparations were differentiated in AFM followed by semi-automated particle recognition, improving the statistical accuracy and precision of absolute height. AF4-MALS and DLS were applied to assess the MW distribution, homogeneity, and stability in solution, which is crucial for attributing an observed bioactivity to a predominant A β aggregate.

A β Aggregate Preparations. To initiate assembly of A β from the monomeric state, NaOH¹² instead of DMSO³¹ was applied to obtain a seedless A β stock solution, which resulted in a higher yield of soluble A β aggregates. Because of the instant aggregation of A β 42, A β 40 was used as monomeric control. Mono remained predominantly monomeric as shown by DLS, AF4-MALS, and AFM. In contrast, freshly dissolved A β 42, frequently used as a monomer control,^{32,33} revealed rapid aggregation and thus was considered as a mixture of nascent A β assemblies comprising LMWO and larger aggregates as described by others.^{14,19,34} PL1 appeared to be similar to ADDL, revealing spheroidal particles with a height of 6 nm and an elution profile in SEC (Figure S4 of the Supporting Information) as described by others.^{31,35} The observed AFM dimensions of PL1 were in line with the MW distribution in AF4-MALS, if spheroidal to disk-shaped A β assemblies are considered.^{11,35} In AF4-MALS, PL1 appeared as continuously distributed A β assemblies with a MW of 73 kDa at peak and thus smaller than ADDL analyzed by SEC-MALS (250–500 kDa at peak^{9,32}). This discrepancy might relate to the improved resolution of AF4 compared to that of SEC, illustrated by the separation of PL1 and PFB (644 kDa at peak) in AF4-MALS. Notably, the Sephadex-75 column focuses aggregates >60 kDa in the void volume as described for protofibrils and ADDL (Figure S4 of the Supporting Information^{9,20,36}). PL2 revealed a bimodal height distribution via AFM with spherical A β assemblies of 2 and 6 nm, with the latter being slightly elongated (38 nm), potentially relating to protofibrillar precursors. Comparable results have been described for early disk-shaped A β assemblies obtained via PBS at 4 °C in single-touch AFM experiments with a height of 2–3 nm and a diameter of 10–15 nm. These aggregates are described as penta- to hexameric A β 42, coexisting with multiple layers of these aggregates, which might reflect PL2-P2.^{11,19} Character-

ization of PFB and FB confirmed published data revealing cross β -sheet containing curvilinear structures of >150 kDa and >1 MDa and 50 nm and several micrometers in length, respectively.^{10,19,36,37}

Metastability of A β Aggregates. The cytotoxic potency of A β aggregates is frequently determined by the sequential characterization of A β aggregate morphology and the biological experiment. The characterization is *prefacto*, and attributing an observed effect to a certain species requires stability under the conditions of the biological experiment.⁸ Here we showed that *prefacto*-characterized A β aggregates are stable, but only if kept in their original environment. Indeed, a change in the ionic environment provoked reorganization of metastable monomeric A β 40 and PL1 aggregates, indicating that applied buffer conditions are highly relevant for not only ab initio aggregation^{8,10,19,38} but also in situ, if bioactivity of preformed A β aggregates is addressed. The provoked aggregation of Mono or PL1 to protofibrillar and fibrillar assemblies by dilution in neurobasal medium most likely explains why comparable neurotoxicity was observed after 48 h. Similarly, in situ A β aggregation has been shown to be prerequisite for neurotoxicity^{39,40} and might be related to a unique cytotoxic potential for each individual species on the pathway to fibrils.^{15,41} The rapid rearrangement in combination with long incubation times challenges the experimentally determined cytotoxic potentials, because structural characterization and the biological experiment are temporally incoherent. To summarize, our setup revealed that all aggregation protocols generated A β aggregates with favored morphology and MW, but no distinct A β species. Notably, A β 42 aggregation in PBS, DMEM, or Ham's F12 resulted in different predominant A β aggregates. Thus, slight changes in aggregation protocols result in A β preparations with variable aggregate compositions and might contribute to controversial results, as exemplified by the interaction of A β with the prion protein.^{32,33,42} Indeed, these examples all used different aggregation conditions while referring to ADDL, originally described by Lambert et al.³¹ All A β aggregates are metastable, and subtle changes in the ionic environment led to reorganization, both *prefacto* and in situ. The importance of the applied ionic environment has generally been underestimated and has to be critically reviewed to allow for comparison of biological effects.

Different Soluble A β Aggregates Mediate Synaptotoxicity by Different Mechanisms. In contrast to neurotoxicity measurements, we chose neuronal binding and hippocampal neurotransmission as surrogate models to assess the bioactivity of a certain A β preparation. Both settings revealed short-term incubations, and aggregate reorganization was negligible. As described by others, Mono⁹ and FB⁴³ did not bind. In contrast, prefibrillar aggregates (PL1, PL2, and PFB) demonstrated a punctate pattern of binding to mature neurons, as shown for ADDL^{27,44–46} and PFB.¹⁸ To test for relevance of A β aggregates on learning and memory, BNT and LTP were investigated separately.^{31,47,48} BNT is dominated by the activation of AMPAR^{28,49} in contrast to the induction of NMDAR-dependent LTP,^{50–52} which requires activation of both AMPAR- and NMDAR-dependent pathways. For the induction of LTP, the high sodium conductivity of AMPAR allows for full depolarization of the synapse required for activation of NMDAR.^{53,54} To attribute A β -related effects on NMDAR-dependent LTP, BNT must not be impaired and vice versa: if BNT is affected, NMDAR-independent pathways are involved. Thus, this setting was suitable for differentiation of

two different synaptotoxic modalities. However, because of the various possibilities for modulating AMPAR and NMDAR,⁵⁵ an impairment of BNT and LTP do not provide evidence of a distinct target of A β . Monomeric and fibrillar A β affected neither BNT nor LTP, supporting the concept that A β monomers and fibrils are virtually inactive.^{18,56} Consistent with the binding experiments, all prefibrillar aggregates affected neurotransmission, although by different mechanisms. PL1 did not affect BNT but inhibited NMDAR-dependent LTP dose-dependently, as described for ADDL,^{57,58} providing further evidence that PL1 and ADDL are equal in size, morphology, and bioactivity by all measures. In contrast to PL1, PL2 and PFB impaired BNT. These preparations rapidly inhibited BNT with nanomolar potency indicative of impaired AMPAR signaling. Similar data for this rapid onset of action on BNT have been obtained for small nascent ThioT-positive A β aggregates¹⁵ and protofibrillar A β preparations.³⁷ Although a conformational change in A β aggregates upon binding to the neuronal matrix cannot be ruled out, it is tempting to speculate that PL2 and PFB are intermediates of the same aggregation pathway and share similar conformational elements. This hypothesis is based on their joint inhibitory effect on AMPAR-dominated BNT and their morphology in AFM, resembling hexameric A β and multiples hereof.^{11,19} Although this hypothesis needs further experimental validation, it seems to be an attractive model for explaining why both preparations inhibited BNT. In addition, the provoked conversion of PL1 to protofibrillar and fibrillar aggregates, which was paralleled by a time-dependent suppression of BNT, indicates a change in the modality of synaptic suppression and thus stresses both the importance of addressing the metastability and the unaffected BNT, before LTP induction. Especially the latter has been discussed recently for the controversial debate about the interaction of ADDL with the prion protein^{32,42} and may explain why Hartley et al.⁵⁶ observed LTP inhibition with cross-linked A β protofibrils, without investigating BNT. Whether inhibition of AMPAR-dependent BNT or NMDAR-dependent LTP represents a distinct mechanism or a mixed effect remains to be investigated, because of the various possibilities of modulating negatively AMPAR and NMDAR. Nevertheless, ADDL (here PL1) has been reported to interact directly with mGluR⁵⁹ or the prion protein,^{32,42} which both critically affect NMDAR function^{43,44,48,60,61} and thus provide a mechanistic model in line with our results for LTP inhibition by PL1. With regard to BNT, several studies described a direct inhibitory action of A β on AMPAR, which could be related to the observed effects, although the aggregation state or morphology was hardly characterized.^{62–65}

A further mechanism, possibly affecting LTP and BNT, is based on the affinity of A β for the neuronal membrane. Indeed, because of its hydrophobicity, A β is able to interact with the lipid bilayer of the neuronal membrane. The consequence of such an interaction could influence the fluidity of the lipid bilayer,^{66,67} change the properties of the membrane via insertion,⁶⁸ or promote the release of membrane lipids. This results in impaired lipid homeostasis⁶⁹ and can lead to the formation of amyloid pores, thus affecting plasma membrane integrity and conductivity.^{70,71} It has been reported that monomeric A β , low-molecular weight aggregates, and oligomeric A β penetrate into the lipid bilayer, resulting in a thinning of the membrane⁷² and an altered structure and fluidity.^{73,74} In addition, it has been shown that A β tends to aggregate further upon binding to the lipid bilayer, because of surface charge and

hydrophobicity.⁷⁵ Thus, the A β peptide promotes structural changes in the neuronal membrane and vice versa. A β aggregates have varying affinities for membrane lipids such as monosialoganglioside GM1, cholesterol, and sphingomyelin, which are predominant in lipid rafts. Given that the local environment of the membrane differs ultrastructurally for lipid rafts, postsynaptic densities and perisynaptic regions, as well as for the local environment of membrane-anchored receptor and structure proteins such as NMDAR, AMPAR, integrins, and PSD95,^{76,77} one could assume that the different A β aggregate species interact both with membrane proteins and with the membrane itself. Because the receptor function is also dependent on a defined structural environment, A β could trigger a concerted action on the membrane, receptors, and related structure and scaffolding proteins. The PL2 presented here might be involved in the AMPAR pathway; however, unspecific depolarization as a result of pore formation could also result in a decreased level of BNT. Nevertheless, PL1 interacts differently, either by direct interaction with the NMDAR pathway or by changing local membrane conditions resulting in the inhibition of NMDAR signaling.

Finally, various A β assemblies, derived from cells, transgenic tissue, or synthetic peptide, have been shown to impair cognition in vivo;⁷⁸ the fate of these assemblies within the brain could not be investigated previously.⁷ Therefore, we aimed for a head-to-head comparison in vitro, where the difference in A β aggregate morphology and fate is accessible, and obtained different synaptotoxic modalities for different A β assemblies. Although the prefibrillar A β preparations were highly potent in impairing neurotransmission, they did not represent a single entity and were metastable, confounding a quantitative assessment of their unique cytotoxic potential. Our study reveals that A β aggregates do not represent distinct entities but rather equivocal distributions in morphology and MW. All assemblies are only metastable, and thus, each manipulation for assessing their specific activity in biological paradigms should avoid prolonged incubation in different environments. This minimizes confounding factors and allows us to address the unique cytotoxic potential of certain A β aggregates. Furthermore, our study shows that different A β aggregates are relevant in AD, because both spheroidal and protofibrillar A β aggregates inhibit hippocampal neurotransmission by different modalities. This challenges the therapeutic approach to target a specific “toxic” A β assembly because A β aggregates reveal synaptotoxicity by various modalities.

■ ASSOCIATED CONTENT

● Supporting Information

AFM particle analysis (Figure S1), A β solubilization (Figure S2), AFM oversegmentation (Figure S3), A β in SDS–PAGE and SEC (Figure S4), A β in TEM, Thio-T, and A11 Dotblot (Figure S5), and A β binding to hippocampal cultures (Figure S6). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

J.M. designed the study, performed the biochemical, biophysical, and biological experiments, maintained the primary

neuronal culture, analyzed the data, and wrote the paper. K.S.K. performed the electrophysiological experiments. D.S. maintained the primary neuronal culture and performed the toxicity experiments. C.S., C.A.F.v.A., B.H., and H.R. supported in writing the paper. L.K. coordinated the study, analyzed the data, and wrote the paper.

Funding

This work was funded by Boehringer Ingelheim Pharma GmbH & Co. KG (CNS Department) and the University of Ulm (Department of Neurology).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Andrew Doig, Kelly Allers, Scott Hobson, Owen Scudamore and Martin Scudamore for carefully reviewing the manuscript.

ABBREVIATIONS

aCSF, artificial cerebrospinal fluid; ADDL, Alzheimer-derived diffusible ligand; AF4, asymmetrical flow field-flow fractionation; AFM, atomic force microscopy; AMPAR, AMPA receptor; A β , amyloid β ; A β O, amyloid β oligomer; BNT, basal neurotransmission; CaMK, Ca²⁺ calmodulin kinase; DIV, days in vitro; DLS, dynamic light scattering; FB, fibril preparation; fEPSP, field excitatory postsynaptic potential; HFIP, hexafluoro-2-propanol; HFS, high-frequency stimulation; LMWO, low-molecular weight oligomer; LTP, long-term potentiation; MALS, multiangle light scattering; Mono, monomer preparation; MTT, dimethylthiazoldiphenyltetrazolium bromide; MW, molecular weight; NMDAR, NMDA receptor; PAGE, polyacrylamide gel electrophoresis; PES, polyethersulfon; PFB, protofibril preparation; PL1/PL2, oligomer aggregate 1/2 preparation; R_h, hydrodynamic radius; SDS, sodium dodecyl sulfate; TEM, transmission electron microscopy.

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