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**Design, characterization, and use of a novel amyloid β -
protein control for assembly, neurotoxicity, and gene
expression studies**

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Running Title: A β aggregation and neurotoxicity control

Abbreviations and Textual Footnotes

Aβ	Amyloid β-protein
AD	Alzheimer's disease
APS	Ammonium persulfate
CD	Circular dichroism
ds cDNA	Double stranded cDNA
TO	Topological overlap
DDI	Double-distilled de-ionized
DIV	Days <i>in vitro</i>
DTT	Dithiothreitol
FBS	Fetal bovine serum
Fmoc	9-fluorenylmethoxycarbonyl
FU	Arbitrary fluorescence units
HC	Hippocampal
HS	Horse serum
LMW	Low molecular weight
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MWCO	Molecular weight cut-off
NGF	Nerve growth factor
NFT	Neurofibrillary tangles
NOS	Nitric oxide synthase
PC12	Rat pheochromocytoma
pI	Isoelectric point

1	PICUP	Photo-induced cross-linking of unmodified proteins
2		
3	P/S	Penicillin/streptomycin
4		
5	RIN	Sample RNA integrity
6		
7		
8	Ru(Bpy) ₃	Tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate
9		
10	SAR	Structure-activity relationship
11		
12	sAβ ₄₀	YHAGVDKEVVFDEGGAEHGLAQKIVRGFGVSDVSMIHNL
13		
14	sAβ ₄₂	YHAGVDKEVVFDEGAGAEHGLAQKIVRGFGVSDVSMIHNL
15		
16	SDS-PAGE	Sodium dodecyl-polyacrylamide gel electrophoresis
17		
18	ThT	Thioflavin T
19		
20	TO	Topological overlap
21		
22	v/v	Volume to volume
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24	WGCNA	Weighted gene co-expression network analysis
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Abstract

A key pathogenic agent in Alzheimer's disease (AD) is the amyloid β -protein ($A\beta$), which self-assembles into a variety of neurotoxic structures. Establishing structure-activity relationships for these assemblies, which is critical for proper therapeutic target identification and design, requires aggregation and neurotoxicity experiments that are properly controlled with respect to the $A\beta$ peptide itself. "Reverse" $A\beta$ or non- $A\beta$ peptides suffer from the fact that their biophysical properties are too similar or dissimilar, respectively, to native $A\beta$ for them to be appropriate controls. For this reason, we used simple protein design principles to create scrambled $A\beta$ peptides predicted to behave distinctly from native $A\beta$. We showed that our prediction was true by monitoring secondary structure dynamics with Thioflavin T fluorescence and circular dichroism spectroscopy, determining oligomer size distributions, and assaying neurotoxic activity. We then demonstrated the utility of the scrambled $A\beta$ peptides by using them to control experiments examining the effects of $A\beta$ monomers, dimers, higher-order oligomers, and fibrils on gene expression in primary rat hippocampal neurons. Significant changes in gene expression were observed for all peptide assemblies, but fibrils induced the largest changes. Weighted gene co-expression network analysis revealed two predominant gene modules related to $A\beta$ treatment. Many genes within these modules were associated with inflammatory signaling pathways.

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Alzheimer’s disease (AD) is the most common late-life neurodegenerative disorder ¹, affecting >5 million people in the United States and >27 million people worldwide ². AD is predominantly a disease of the aged, as >95% of patients are ≥65 years of age. Patients generally present initially with memory deficits, which then are followed by an inexorable decline in executive function and other mental abilities. AD is characterized histopathologically by intraneuronal neurofibrillary tangles (NFTs) formed by the microtubule-associating protein tau and by extraneuronal amyloid plaques formed by the amyloid β-protein (Aβ) ¹. Two alloforms of Aβ predominate in humans, Aβ₄₀, which contains 40 amino acids, and Aβ₄₂, which contains 42 amino acids ³. Cerebrovascular amyloid deposits have an abundance of Aβ₄₀, whereas the Aβ component of parenchymal plaques contains primarily Aβ₄₂ ^{4,5}. Plaque formation is associated with development and progression of AD ⁶. For this reason, myriad studies have sought to elucidate the pathway(s) of Aβ fibril formation so that therapeutic agents could be targeted to those steps critical in the process. In doing so, an array of smaller Aβ assemblies, including low-order oligomers, annuli, globulomers, and protofibrils has been revealed ⁷. This has led quite naturally to the determination of structure-activity relationships (SAR), which has shown that Aβ oligomers appear to be key neurotoxins ⁷⁻⁹.

We hypothesize that a neuron’s ability to protect itself from the effects of Aβ oligomers, and other Aβ assemblies, is determined by age-related changes in the transcription of specific genes. As a first step to testing this hypothesis, we sought to use gene microarrays to determine gene expression changes induced in embryonic primary hippocampal (Hc) neuron cultures by monomers, oligomers, protofibrils, or fibrils formed by Aβ₄₀ or Aβ₄₂. However, a necessary prerequisite for performing these experiments were appropriate Aβ₄₀ and Aβ₄₂ control peptides, which would allow differentiation of gene expression changes due to the effects Aβ assemblies from those changes due to generic peptide:neuron interactions. We report here the design of such peptides, comparative

analysis of their structures and assembly behaviors, and their initial use in gene microarray experiments.

Experimental Procedures

Chemicals and Reagents

Unless specified otherwise, all chemicals and sera were obtained from Sigma-Aldrich (St. Louis, MO) and were of the highest purity available. Tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate (Ru(Bpy)₃), ammonium persulfate (APS), Novex® 10-20% Tricine SDS gels, Neurobasal A media, B27 growth factor, glutamine, Penicillin/Streptomycin Solution were purchased from Invitrogen (Carlsbad, CA). Trypsin-EDTA (2.5% w/v) was obtained from ATCC (Manassas, VA). Scalpels were purchased from Sigma Aldrich. All solutions were prepared in double-distilled de-ionized (DDI) water produced using a Milli-Q system (Millipore Corp., Bedford, MA).

Peptide Design

Word scrambler software (<http://www.lerfjhx.com/scrambler>) was used to randomly permute the Aβ amino acid sequence. We then used Kyte-Doolittle analysis¹⁰ with a window size of 9 (<http://web.expasy.org/protscale/>) to determine the hydropathy profiles of these permuted sequences (Fig. 1). The scrambled Aβ peptides below are identical to their cognate native Aβ peptides in length, pI, and amino acid composition, but they are not amphipathic.

Scrambled Aβ40 (sAβ40): YHAGVDKEVVVFDEGGAEHGLAQKIVRGFGVSDVSMIHNL

Scrambled Aβ42 (sAβ42): YHAGVDKEVVVFDEGAGAEGHGLAQKIVRGFGVSDVSMIHINL

Peptide Synthesis

All peptides were synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry, purified by

reverse phase-high performance liquid chromatography, and characterized by mass spectrometry and amino acid analysis, as described previously [7]. Quantitative amino acid analysis and mass spectrometry yielded the expected compositions and molecular weights, respectively, for each peptide. Purified peptides were stored as lyophilizates at -20°C.

Preparation of Low Molecular Weight (LMW), Oligomeric, and Fibrillar A β

Low molecular weight (LMW) A β comprises a mixture of A β monomers and dimers that exist in equilibrium ¹¹). Chemically stabilized oligomers were prepared using the method of Photo-Induced Cross-linking of Unmodified Proteins (PICUP), as described [10, 11]. Briefly, A β lyophilizates were dissolved in 10 mM sodium phosphate, pH 7.5, at a concentration of 80 μ M and cross-linked by mixing 36 μ L of protein solution with 2 μ L of 2 mM Ru(Bpy)₃ and 2 μ L of 40 mM APS. The final A β :Ru(Bpy)₃:APS molar ratios were 0.72:1:20. The mixture was irradiated for 1 s with visible light from a 150 W fiber optic illuminator (model 170-D, Dolan-Jenner, Lawrence, MA, USA), after which the reaction was quenched with 2 μ L of 1 M dithiothreitol (DTT). Cross-linking reagents were removed using a Zeba Spin Desalting Column, 7K MWCO (Thermo Fisher Scientific, Rockford, IL). SDS-PAGE and silver staining were used to determine the frequency distribution of A β oligomers. This was accomplished by diluting the PICUP-treated A β to a concentration of 40 μ M. Twenty-one μ L of each cross-linked sample mixed with 21 μ L of 2 $\times$ SDS sample buffer was boiled for 10 min. Thereafter, 8 μ L of each boiled cross-linked sample was electrophoresed on a 10-20% gradient Tricine gel (Invitrogen, Carlsbad, CA) and visualized by silver staining (SilverXpress, Invitrogen). Cross-linked A β ₄₀ and A β ₄₂ have distinct oligomer distributions that differ from distributions obtained in the absence of cross-linking (see Results and ¹²). These distributions also differ from those obtained by preparation of oligomers using methods that do not stabilize the non-covalent interactions mediating the oligomerization process ¹³⁻¹⁷. Non-cross-linked samples were used as

controls in each experiment. Fibrils were prepared by gently agitating a 1 mg/mL solution of LMW A β in 10 mM sodium phosphate, pH 7.5, at 37°C for 1 week. Non-fibrillar assemblies were removed by filtration using a 50 kDa molecular weight cut-off (MWCO) centricon filter. We note that the results obtained using the four different classes of A β assemblies prepared here are valid for determination of SAR, as the structures of each class are known. However, the relationships determined here may, or may not, match those determined in other systems because the A β preparations differ.

Circular Dichroism

A β samples were prepared in 10 mM sodium phosphate, pH 7.5, at a concentration of 50 μ M and incubated at 37°C in 1-mm path length quartz cuvettes (Hellma, Forest Hills, NY). CD spectra were acquired using a J-810 spectropolarimeter (JASCO, Tokyo, Japan). Following temperature equilibration, spectra were recorded at 22°C from 195–260 nm at 0.2 nm resolution with a scan rate of 100 nm/min. Spectra were acquired immediately after sample preparation and then periodically for two weeks thereafter. Eight scans were acquired and averaged for each sample at each time point. Buffer spectra were subtracted from appropriate experimental spectra prior to data analysis.

Thioflavin T (ThT) Assay

ThT fluorescence was used to monitor β -sheet formation. Peptides were incubated at a final concentration of 50 μ M in 10 mM sodium phosphate, pH 7.5. All assay solutions contained 20 μ M ThT. One-hundred μ L of each sample were placed into each of three wells (triplicates) of a white plastic, clear-bottomed, 96-well microtiter plate (Nalge Nunc International, Rochester, NY) and then the plate was incubated at 37°C without agitation. The plate was removed from the incubator and placed in a Synergy HT plate reader (Bio-Tek Instruments, Winooski, VT) for fluorescence measurements. Excitation and emission wavelengths/slit widths were 450/50 and 485/20 nm,

1 respectively. Results were plotted using KaleidaGraph, v. 4.0.4 (Synergy Software, Reading, PA).
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5
6 *Neurotoxicity Assay*
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9 To determine the effects of A β on MTT metabolism, rat pheochromocytoma (PC12) cells were
10 cultured in 75 cm² Canted Neck Flasks (Corning Inc., Corning, NY) in F-12K medium containing 10%
11 (v/v) HS, 2.5% (v/v) fetal bovine serum (FBS), 100 units/mL penicillin, 0.1 mg/mL of streptomycin, and
12 25 μ g/mL amphotericin B at 37°C in 5% (v/v) CO₂ in air. The cells were passaged at least 4 times
13 before use. To prepare the PC12 cells for toxicity analysis, the medium was removed and the cells
14 were washed once gently with F-12K medium, containing 0.5% (v/v) FBS, 100 units/mL penicillin, 0.1
15 mg/mL streptomycin, and 25 μ g/mL amphotericin B. A cell suspension was then made with this
16 medium supplemented with 100 μ g/mL nerve growth factor (NGF). Cell concentration was
17 determined by Trypan blue staining, after which cells were plated at a concentration of 30,000
18 cells/well (80 μ L of total volume per well) in Costar 96-well white clear-bottom plates (Corning,
19 Corning, NY). The NGF-induced differentiation of the cells was allowed to proceed for 48 h. To
20 perform the toxicity assays, 50 μ M of each A β assembly was prepared in F-12K medium containing
21 0.5% (v/v) FBS, 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 25 μ g/mL amphotericin B.
22 Twenty μ L aliquots of the A β solution were added to the wells to yield a final A β concentration of 10
23 μ M, which is within the 1-100 μ M concentration regime typically used in MTT assays with PC12 cells
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18-21. The cells then were incubated for 17 h, after which 15 μ L of CellTiter 96® Non-Radioactive Cell
Proliferation Assay MTT solution (Promega, Madison, WI) was added to each well and the plate was
incubated at 37°C in the 5% CO₂ incubator for 3 h. Thereafter, the cells were lysed by the addition of
100 μ L of solubilization solution (Promega, Madison, WI) and the plate was stored overnight at 37°C
in 5% CO₂ incubator. The next day, MTT reduction was determined by measuring absorption at 570
nm (corrected for background absorbance at 630 nm) with a Synergy HT plate reader (Bio-Tek

Instruments, Winooski, VT). The negative control was F12-K medium alone. The positive toxicity control was 1 μ M staurosporine. Eight to twelve replicates were used for each treatment group and the data were reported as mean $\pm$ SE. Percent viability $V = (1 - (A_{A\beta} - A_{\text{medium}})/(A_{\text{full kill}} - A_{\text{medium}})) \times 100$, where $A_{A\beta}$, A_{medium} , and $A_{\text{full kill}}$ were absorbance values from $A\beta$ containing samples, negative control (buffer equivalent with medium), and 1 μ M staurosporine alone, respectively.

Cell Culture and Treatment with A β

The method of Meberg²² was used to prepare fresh primary neurons. Embryonic day 18 (E18) rats were sacrificed and the hippocampi were carefully excised using curved forceps. The hippocampi were transferred to Petri dishes, containing 5 mL of Neurobasal A/B27 media (Neurobasal A media supplemented with 2% (v/v) B27), where they were cut into $\approx$ 1 mm pieces with a sterilized razor blade. Enzymatic digestion of extracellular matrix proteins was accomplished by trypsin digestion (2 mL of 10 $\times$ trypsin (2.5% w/v) were added to the suspension for 1h at 37°C in a 5% (v/v) CO₂ incubator). This solution was gently inverted every 5 min. The media was removed and the cells placed in fresh, warm Neurobasal A/B27 media where the cells were dissociated by trituration (20 $\times$) with a fire-polished Pasteur pipette. Cell concentrations were quantified using a hemocytometer and 1:1 mixture of a cell culture aliquot:Trypan blue. Cells were plated on Petri dishes or 12-well culture plates previously coated with poly-D-lysine (for at least 2 h) at a concentration of 3×10^5 viable cells/mL. The medium was pre-warmed (37°C) Neurobasal A/B27 media supplemented with 0.5 mM glutamine, 10% (v/v) horse serum (HS), and 10 μ g/mL penicillin/streptomycin (P/S). After 2 h incubation in a 37°C, 5% CO₂ incubator, the medium containing unattached cells was removed and fresh medium was added. Cells were allowed to adhere, stabilize metabolically, and grow projections, which typically occurred by 7 days *in vitro* (DIV) (Fig. S1). We note that this protocol has been found to yield almost pure cultures of neurons, although some (<0.5%) glial cells may be

1 present ²³. Aβ treatment was initiated by removing half the medium and replacing it with an equal
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4 volume of fresh medium containing a particular Aβ assembly at a protein concentration of 20 μM.
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6 After 24 h, the RNA from these neurons was extracted and used in array analysis.
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11 *RNA Extraction, Quantification, and Quality Analysis*
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14 Total RNA was obtained using an RNeasy kit (Qiagen, Valencia, CA). The concentration of the RNA in
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16 the extracts was determined using a NanoDrop ND-1000 spectrophotometer (Wilmington, DE). The
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18 RNA quality was assessed with a RNA Nano 6000 LabChip® (Agilent Technologies, Inc., Santa Clara,
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20 CA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). Sample RNA integrity number
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22 (RIN) was consistently >9 (RIN values above 8 are considered suitable for array analysis) ²⁴.
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29 *RNA Amplification, Labeling, and Hybridization*
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32 Total RNA was processed at the UCLA Neuroscience Genomics Core (UNGC) and quantified using a
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34 Ribogreen fluorescent assay. Intersample RNA concentrations were normalized to 10 ng/μL prior to
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36 amplification. Amplified and labeled cRNA was produced using the Illumina-specific Ambion
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38 TotalPrep kit based on a T7-based linear amplification method ²⁵. First and second strand cDNA was
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40 produced using the Ambion kit and purified using a robotic assisted magnetic capture step. Briefly,
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42 this step employs the use of an oligo(dT) primer coupled to the phage T7 RNA polymerase promoter
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44 to prime the synthesis of the complementary DNA by reverse transcription with reverse transcriptase
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46 of the poly(A) tail and the associated RNA component of total RNA. Double stranded cDNA (ds
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48 cDNA) is produced by RNase H degradation of the original poly A containing RNA followed by strand
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50 synthesis with DNA polymerase I. In vitro transcription to synthesize cRNA generates multiple copies
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52 of biotinylated cRNA from the double-stranded cDNA templates. Single and double amplification
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54 rounds can produce two thousand-fold and one million-fold increases of transcript from the initial
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material with typical yields in excess of 1.5 µg. After a second Ribogreen quantification and normalization step, amplified and labeled cRNA was hybridized overnight at 58°C to the RatRef-12 Expression BeadChip (Illumina, San Diego, CA), containing 22,523 probes per array. Hybridization was followed by washing, blocking, staining, and drying on the Little Dipper processor. Array chips were scanned on the Beadarray reader, and expression data was extracted and compiled using BeadStudio software (Illumina).

Microarray Data and Network construction based on WGCNA

The raw data from the microarray was scaled to the same average intensity and normalized using a quantile normalization method²⁶. Raw data was analyzed using Bioconductor packages (www.bioconductor.org). Quality was assessed by determining the inter-array Pearson correlation coefficient. Clustering based on top variant genes was used to assess overall data coherence. We performed two distinct analyses: 1) differential expression analysis, and 2) weighted gene co-expression network analysis (WGCNA). Contrast analysis of differential expression was performed using the LIMMA package²⁷. After linear model fitting, a Bayesian estimate of differential expression was calculated. Data analysis was aimed at identifying transcriptional changes induced by the various treatments. The threshold for statistical significance was set at $p < 0.005$. Gene ontology and pathway analyses were carried out using the "Database for Annotation, Visualization and Integrated Discovery" (<https://david.ncifcrf.gov/>) and "Ingenuity Pathway Analysis" (www.ingenuity.com). We then conducted WGCNA, a systems biology approach used to identify networks of co-expressed genes in relation to phenotypic data, using the R package, as previously described^{28, 29}. Briefly, correlation coefficients were constructed between expression levels of the most variable probes ($n=6,274$) and a connectivity measure, topological overlap (TO), was calculated for each gene by summing the connection strength with other genes. Genes were then clustered based on their TO,

1 and groups of co-expressed genes (modules) were identified. Each module was assigned a color, and
2
3 the first principal component (eigengene) of a module was extracted from the module and
4
5 considered to be representative of the gene expression profiles in a module³⁰. To depict the pairwise
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7 relationships between genes, VisANT software (available at <http://visant.bu.edu>) was employed.
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11 Approximately 500 pairs of genes with the highest intra-modular connectivity are shown.
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Results

Design and characterization of control peptides

Key factors relevant to the biophysical and biological behavior of A β are its: (1) length; (2) pI; (3) amino acid composition; and (4) sequence. These factors control A β assembly (e.g., into oligomers, protofibrils, fibrils) and the interactions between the peptide assemblies and neurons that affect gene expression, cell metabolism, electrical activity, and viability. We reasoned that an ideal control peptide for experiments in which A β assembly and biological activity are studied should have the same length, pI, and amino acid composition as the native peptide. Reverse peptides meet this requirement^{29, 31-33}, but they also maintain identical hydropathy profiles characterized by marked amphipathicity. This means that peptide:neuron interactions involving factors such as partitioning of hydrophobic peptide segments into membranes or formation of membrane pores might be similar for both native and reverse peptides, which would prevent determination of gene expression differences induced by peptide treatment *per se* versus differences linked specifically to A β . To eliminate this problem, we designed scrambled A β ₄₀ (sA β ₄₀) and A β ₄₂ (sA β ₄₂) sequences having hydropathy profiles with as little divergence as possible from an hydropathy statistic of 0 (Fig. 1; see Methods). We then chemically synthesized sA β ₄₀ and sA β ₄₂ and compared their biophysical properties with those of their cognate native forms to demonstrate that their properties were distinct and thus that the scrambled peptides could indeed be appropriate controls.

Thioflavin T (ThT) fluorescence

Temporal changes in Thioflavin T (ThT) fluorescence were used to reveal β -sheet formation for freshly prepared, aggregate-free A β incubated at 37°C. A β ₄₀ displayed a monotonic increase in ThT fluorescence that reached a plateau after $\approx$ 200 h (Fig. 2A, black circles). The ThT signal increased 15-fold during the assay, demonstrating that β -sheet formation occurred. In comparison, sA β ₄₀ showed a relatively small (2-fold) increase in ThT fluorescence that reached a plateau after $\approx$ 60 h (Fig. 2A, white circles). A β ₄₂ displayed a rapid increase in ThT fluorescence that reached a plateau of $\approx$ 520 FU after $\approx$ 50 h (Fig. 2B, black circles). In comparison, sA β ₄₂ showed a rapid, transient increase in ThT fluorescence within 10 h that decreased to, and remained at, baseline levels ($\approx$ 30-40 FU) after $\approx$ 50 h (Fig. 2B, white circles).

Circular dichroism (CD) spectroscopy

We used CD spectroscopy to determine secondary structure dynamics. A β ₄₀ initially displayed a spectrum characterized by a prominent minimum at $\approx$ 198 nm and increasing ellipticity toward higher wavelengths (Fig. 3A). Inflections were observed at $\approx$ 215 and $\approx$ 230 nm. This spectrum is characteristic of statistical coil secondary structure^{34, 35}. The spectral shape changed progressively during 14 d of incubation, yielding a final spectrum displayed a peak of ellipticity at $\approx$ 195 nm and a minimum at $\approx$ 215 nm, consistent with β -sheet. In contrast, sA β ₄₀ displayed spectra consistent with statistical coil throughout the incubation period (Fig. 3B). Qualitatively similar data were observed in studies of A β ₄₂. The initial A β ₄₂ spectrum displayed statistical coil characteristics, which changed over time to a classical β -sheet form (Fig. 3C). sA β ₄₂ displayed spectra consistent with statistical coil secondary structure throughout the incubation period (Fig. 3D).

1 *Oligomerization*
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4 The oligomerization of the different A β peptides was monitored by zero-length, photochemical
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6 cross-linking (PICUP) followed by SDS-PAGE and silver staining (Fig. 4). In the absence of cross-
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8 linking, A β ₄₀ displayed almost exclusively monomers (Fig. 4, lane 1). After cross-linking, the A β ₄₀
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10 oligomer distribution comprised monomers through hexamers (Fig. 4, lane 2), with trimer exhibiting
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12 the most intense band. A β ₄₂ displayed monomers and trimers (Fig. 4, lane 3). After cross-linking,
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14 A β ₄₂ produced an oligomer distribution comprising monomers through octamers (Fig. 4, lane 4),
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16 with nodes at dimer and pentamer/hexamer (Fig. 4, black arrowhead). SA β ₄₀ displayed monomers,
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18 but no higher molecular weight bands (Fig. 4, lane 5). Four prominent bands were observed after
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20 cross-linking of sA β ₄₀, which we interpret to be dimer through pentamer (Fig. 4, lane 6). A faint
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22 hexamer band also was seen. A similar pattern of bands was seen with sA β ₄₂ (Fig. 4, lane 8). We note
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24 that the mobilities of the bands in the two cross-linked scrambled peptides differed from the
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26 mobilities of the respective bands in the wild type A β ₄₀ (cf. lanes 2 and 6) and A β ₄₂ samples (cf. lanes
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28 4 and 8). We note, in addition, that the cross-linking of the scrambled peptides was particularly
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30 efficient relative to the wild type peptides (cf. monomer band intensities of lanes 5 and 7 with those of
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32 lanes 6 and 8; and monomer intensities of lanes 1 and 3 with those of lanes 2 and 4). Taken together
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34 with the results of the CD experiments, these data suggest that the scrambled peptides interact
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36 rapidly to form oligomers but that these oligomers do not possess the ordered secondary structure
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38 elements (e.g., β -sheet) found in A β ₄₀ and A β ₄₂.
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51 *Toxicity*
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54 To study the biological activity of the scrambled peptides and how it related to the activities of LMW,
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56 oligomeric (oligomers stabilized by cross-linking; XL), and fibrillar A β assemblies, we studied the
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58 effects of the different A β preparations on cell metabolism using MTT assays ³⁶. To do so, rat
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pheochromocytoma (PC12) cells were treated with sA β , LMW A β , cross-linked A β , or fibrillar A β for 24 h at 37°C and then the ability of the cells to metabolize MTT was measured (Fig. 5). The metabolic activity of cells treated with LMW A β ₄₀ and LMW A β ₄₂ was 73% and 83% of the buffer control, respectively, demonstrating modest cytotoxic activity for these A β assemblies. Cross-linked A β ₄₀ and A β ₄₂ produced similar toxic effects. Fibrils of A β ₄₀ and A β ₄₂ were significantly more toxic, reducing MTT metabolism by 63% and 45%, respectively. Only the sA β ₄₀- and sA β ₄₂-treated cells displayed no significant metabolic effects. It has been reported that oligomeric forms of A β are more toxic than fibrils¹³. The most likely explanation for the modest toxicity displayed by the oligomers used here is that their structures differ from those used by Dahlgren *et al.*, which were not stabilized by covalent cross-linking.

Effects of A β assemblies on neuronal gene expression

Our goal in designing sA β ₄₀ and sA β ₄₂ was to control experiments determining structure-activity relationships. The prior results showed that these peptides did not share fibril formation (ThT) or secondary structure (CD) dynamics, oligomerization patterns, or neurotoxic activity (MTT) with their wild type analogues, demonstrating achievement of the goal. We thus used these peptide to control experiments monitoring changes in neuronal gene expression induced by exposure of rat primary Hc neurons to: (1) LMW A β (predominately monomers and dimers); (2) low-order, stable, A β oligomers; or (3) A β fibrils. These species represent three major classes of A β assemblies with potential relevance to AD⁷. Primary rat Hc neurons were incubated for 24 h with each of four different types of A β ₄₀ or A β ₄₂ peptides—LMW, cross-linked oligomers, fibrillar, or scrambled. RNA then was harvested from each treatment group and hybridized onto rat microarray chips. First, analysis of average signal intensity differences among genes allowed us to make comparisons between different treatment groups (Fig. 6, Table S1). The largest numbers of gene changes occurred with fibril treatment,

consistent with the relatively large toxicity of fibrils observed in the MTT assays groups. The total number of changes observed in the fibril-treated groups varied from ≈ 1050 -1600, with similar ($\pm 10\%$) numbers of genes on average showing increased and decreased expression. The largest overall gene expression changes were seen in A β_{42} fibrils vs. control-treated neurons, where 860 and 735 genes are under- and over-expressed, respectively. In comparison, A β_{42} fibrils vs. sA β_{42} -treated neurons showed 671 and 663 genes under- and over-expressed, respectively. The relative difference in differential gene expression between these two groups may be explained in part by sA β_{42} alone, which results in under- and over-expression of 47 and 59 genes, respectively.

We also observed a large number of gene expression changes for cross-linked A β_{40} , which demonstrated ~ 400 gene changes versus sA β_{40} . One interesting feature of the dataset was the relatively low number of changes observed with cross-linked A β_{42} compared to the numbers observed with both A β_{40} and A β_{42} fibrils or cross-linked A β_{40} . Clustering based on the overall differences in gene expression revealed that neurons treated with fibrillar A β cluster separately from other treatment groups (Fig. 7A). Furthermore, neurons treated with cross-linked A β_{40} clustered separately from all other treatment groups. In general, the dendrogram branching pattern suggests that the treatment groups with the greatest magnitude of gene changes cluster separately from those with fewer than 400 gene changes.

Gene ontology analysis of the top differentially expressed genes observed in cells treated with A β_{42} fibrils vs. sA β_{42} revealed overrepresentation of transcripts generally related to inflammatory response (including response to stimulus and other organism), regulation of cell proliferation, and programmed cell death (Figure 7B). Transcripts included in these categories were mostly up regulated, supporting the notion that treatment with some A β assemblies induces an inflammatory response in cultured neurons, which can be associated with cell death.

Network construction and modular organization

Next, we performed weighted gene co-expression network analysis (WGCNA) ²⁹ to elucidate the complex, high-order relationships among gene expression levels. Networks were constructed based on gene topological overlap (TO), which considers the level of correlation between two genes and their degree of shared correlation within the network ²⁹.

Seven thousand of the most variable genes were clustered using TO as a similarity metric, where similarly co-expressed probes clustered together. Distinct groups of clustered genes, termed “modules”, were then defined using an algorithm that analyzes branching pattern ³⁰, and a color was assigned to each module (Fig. S2). We then correlated for each module the module eigengene (ME), a weighted summation of expression profiles of all the transcripts in a given module ³⁷, with the presence or absence of A β treatment. The orange and yellow modules, which are independent from one another, showed the greatest correlation with A β ₄₀ and A β ₄₂ fibril treatment among the total of 13 modules defined. As expected, in each of these modules, the largest number of gene expression changes was seen for neurons treated with fibrillar A β ₄₀ or fibrillar A β ₄₂ (see Fig. 6). However, each module had distinct gene ontologies. The orange module showed an overrepresentation of genes involved in extracellular matrix formation and the yellow module an overrepresentation of transcripts involved in inflammation and responses to cytokines.

The network position of a particular gene was then determined by calculating the ME-centered connectivity. Those transcripts with the highest levels of connectivity (i.e., correlation with the ME) were designated as hub genes. Top hub probes for the orange module included *Ogn*, *LOC311722*, *LOC498662*, *Lox*, *MGC72614*, and *Bambi* (Fig. 8). *Ogn* is the gene for the proteoglycan osteoglycan. *LOC311722*, *LOC498662*, and *MGC72614* are yet uncharacterized genes. *Lox* encodes lysyl oxidase, which is an extracellular copper enzyme that catalyzes the cross-linking of structural proteins such as collagen and elastin. *Bambi* is transmembrane glycoprotein known to negatively regulate

transforming growth factor- β ³⁸. Network visualization of the yellow module reveals six hub genes: *LOC497812*, *Isg12(b)*, *Ifit1*, *RT1-Da*, *Cxcl11*, and *Rtp4*. (Fig. 9). *LOC497812* is an uncharacterized gene. *Isg12(b)*, *Ifit1*, and *Rtp4* are proteins responsive to interferon- γ (IFN- γ). *RT1-Da* is a major histocompatibility (MHC) class II molecule. *Cxcl11* is an inflammatory cytokine.

Discussion

We have hypothesized that a central etiologic mechanism of AD is age-dependent alteration in neuronal gene expression in response to specific A β assemblies. To test this hypothesis, it was necessary to control gene expression experiments for effects caused simply by the interaction of peptides *per se* with neurons, as distinguished from effects induced by particular A β conformers or assemblies (monomers, oligomers, protofibrils, or fibrils). First principles argued that an ideal control would have an amino acid composition identical to that of the native peptide, and therefore identical molecular weight, length, and pI, but *not* share conformational features or assembly properties. We have shown, using a combination of Kyte-Doolittle analysis, ThT fluorescence, CD, photochemical cross-linking and SDS-PAGE/silver staining, and MTT assays, that the scrambled peptides sA β ₄₀ and sA β ₄₂ did not share hydropathy profiles, conformational dynamics, assembly kinetics, or neurotoxic activity with their cognate wild type peptides. These data validated the use of the scrambled peptides as controls, enabling us to begin testing our hypothesis about A β -assembly dependent effects on neuronal gene expression.¹

¹ We are aware of two other sequences of scrambled A β ₄₀ and A β ₄₂ (available from commercial sources (Table S2)). Class I peptides have been used in studies of A β -induced expression of BACE1 mRNA in primary neuronal cultures ³⁹, intracellular rod formation in primary hippocampal neurons ⁴⁰, ligand blotting to APP ⁴¹, and clearance of A β through the blood-brain barrier ⁴². Class II peptides have

We began first by studying the effects of A β monomers and dimers, oligomers, and fibrils on gene expression using embryonic rat primary Hc neurons. We did so to establish how A β assembly types thought to be important in disease causation affected "young" neurons. These studies are a necessary prerequisite for later work using adult neurons. Our analysis revealed a gradient of gene expression changes induced by specific A β assemblies, with the largest magnitude of changes induced by A β ₄₀ and A β ₄₂ fibril treatment. The dominant ontology categories affected by these treatments are related to activation of inflammatory pathways. WGCNA also identified two co-expression modules correlated with A β ₄₀ and A β ₄₂ fibril treatment that were related to extracellular matrix and inflammation. These results are consistent with recent reports that transforming growth factor- β 1 blocks CCL5, which is a chemokine that mediates the chemotaxis of microglia towards A β aggregates ⁴⁴, and that increased expression of argininosuccinate synthetase (the rate-limiting enzyme in the metabolic pathway for the conversion of L-citrulline to L-arginine, which is a substrate for all isoforms of nitric oxide synthases (NOS) ⁴⁵) and inducible NOS (iNOS) is observed in the cortices of AD patients ⁴⁶. NO appears to play a key role in inflammatory processes in the CNS, including both neurotoxic and protective ⁴⁷. In rat mixed neuronal-glial cultures, A β induced the expression of iNOS and argininosuccinate synthetase with a corresponding increase in inflammatory cytokines such as interleukin-1 β and tumor necrosis factor- α ⁴⁶. The observation of

been used in studies of astrocyte activation ⁴³. No systematic studies of the structures and assembly dynamics of these two classes of peptides were reported. EM studies did show that class I scrambled A β ₄₀ and A β ₄₂ did not form "appreciable high molecular weight aggregates" after 1 or 6h when examined by EM ³⁹ and CD spectra obtained immediately after preparation of class II scrambled [Iodo-tyrosyl10]A β ₄₀ were identical to those obtained from wild type [Iodo-tyrosyl10]A β ₄₀ ⁴². Aggregates were formed by class II scrambled A β ₄₂ and these aggregates caused modest astrocyte activation ⁴³.

1 significant cytokine expression is especially interesting in light of recent evidence that the
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4 chemokine CX₃CL₁ (fractalkine) is implicated in the progression and severity of AD-like pathology in
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6 mice ^{48, 49} and that knockout of its receptor CX₃CR₁ protects against Aβ-induced neurotoxicity in
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Supporting Information Available

Table S1, Average signal intensity differences among genes after treatment with specific A β assemblies; **Table S2**, Commercially available scrambled A β peptides; **Fig. S1**, Phase-contrast microscopy image of primary hippocampal neurons after 7 days in culture; **Fig. S2**, Relationship between module eigengene (first principal component, corresponding to the weighted summation of expression across all the probes included in a given module) and treatment (A β assembly type).

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Figure Legends

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Figure 1. Hydropathy profiles for A β ₄₂, reverse A β ₄₂ (rA β ₄₂), and scrambled A β ₄₂ (sA β ₄₂). Abscissa shows position of center of hydropathy window of 9 amino acids. Ordinate is hydropathy metric that denotes hydrophobicity as positive and hydrophilicity as negative.

Figure 2. Time-dependence of ThT fluorescence. (A) A β ₄₀ (black circles) and sA β ₄₀ (white circles) were incubated at 37 °C and Thioflavin T (ThT) fluorescence was determined periodically. (B) A β ₄₂ (black circles) and sA β ₄₂ (white circles) was studied in an identical manner. Fluorescence is presented in arbitrary units (FU). Curve fits are included only for ease of comparison between curves. A minimum of three replicates was done for each sample.

Figure 3. Circular dichroism spectroscopy. Secondary structure dynamics of WT vs. sA β were determined using CD. Spectra are: (A) A β ₄₀, (B) sA β ₄₀, (C) A β ₄₂, and (D) sA β ₄₂. Spectra were acquired at times: 0 h (white circles), 24 h (black circles), 120 h (white triangles), 240 h (black triangles), and 340 h (white squares). A minimum of three replicates was done for each sample.

Figure 4. Oligomer size distributions. PICUP, SDS-PAGE, and silver staining were used to determine the oligomer size distribution of freshly prepared A β ₄₀, A β ₄₂, sA β ₄₀, and sA β ₄₂. Lane 1, non-cross-linked A β ₄₀; Lane 2, cross-linked A β ₄₀; Lane 3, non-cross-linked A β ₄₂; Lane 4, cross-linked A β ₄₂; Lane 5, non-cross-linked sA β ₄₀; Lane 6, cross-linked sA β ₄₀; Lane 7, non-cross-linked sA β ₄₂; Lane 8, cross-linked sA β ₄₂. The results are representative of those obtained in each of three independent experiments. Numbers to the right of lanes signify oligomer order (number of monomers). Black arrowhead indicates an intensity node at pentamer/hexamer.

Figure 5. Toxicity of different A β assemblies. MTT assays were performed on differentiated PC12 cells using 10 μ M final concentrations of LMW A β ₄₀, LMW A β ₄₂, cross-linked A β ₄₀, cross-linked A β ₄₂, fibrillar A β ₄₀, fibrillar A β ₄₂, sA β ₄₀, and sA β ₄₂. Standard errors were derived from 8-12 repetitions of each experiment. Toxicity measurements were normalized to buffer control. The statistical significance of the differences between the mean of LMW A β ₄₀ and the means of XL A β ₄₀ oligomers, A β ₄₀ fibrils, or scrambled A β ₄₀ is indicated by p-values, as follows: *, $p < 0.05$; **, $p < 0.005$, and $p < 0.0005$. p-values for XL A β ₄₂ oligomers, A β ₄₂ fibrils, or scrambled A β ₄₂ are represented by the same symbols but were calculated relative to the mean of LMW A β ₄₂. ND means "not done," and NS means "not significant."

Figure 6: Bar plot representing the number of down regulated (green) and up regulated (red) genes in each comparison. Complete gene lists are in Table S1.

Figure 7: (A) Heat map depicting gene expression changes at each of the comparisons ($p < 0.005$, Bayesian t -test). Each row corresponds to one probe, and each column corresponds to one comparison. Up regulated genes are in red and down regulated genes are in green. The color intensity corresponds to the magnitude of the change in expression level (probes are listed in Supplementary Table). Both comparisons and probes are clustered by similarity (represented by the cluster dendrograms on top and side, respectively). Comparisons are numbered as follows: 1) LMW A β ₄₂ vs. sA β ₄₂; 2) LMW A β ₄₀ vs. sA β ₄₀; 3) cross-linked A β ₄₂ vs. sA β ₄₂; 4) cross-linked A β ₄₀ vs. sA β ₄₀; 5) A β ₄₀ fibrils vs. sA β ₄₀; 6) A β ₄₂ fibrils vs. sA β ₄₂. (B) Over-represented gene ontology (GO) categories among differentially expressed genes in A β ₄₂ fibrils vs. sA β ₄₂ (the proportion of down regulated DE genes is in green and the proportion of up regulated DE genes is in red) sorted by $-\log_{10}(p \text{ value})$. A $-\log(p \text{ value})$ of 1.3 corresponds to an over-representation p value of 0.05.

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Figure 8. Network visualization of the orange module. This plot represents the strongest connections, based on topological overlap (TO), within the orange module and highlights the central most connected genes, or hubs, within the network. The hub genes, which are highlighted with red circles, are Ogn, LOC311722, LOC498662, Lox, MGC72614, and Bambi.

Figure 9. Network visualization of the yellow module. This plot represents the strongest connections, based on TO, within the yellow module and highlights the central most connected genes, or hubs, within the network. The hub genes, which are highlighted with red circles, are LOC497812, lsg12(b), Ifit1, RT1-Da, Cxcl11, and Rtp4.

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Fig. 1

Fig. 2A

Fig. 2B

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ACS Paragon Plus Environment

Fig. 4

Fig. 5

Fig. 7B

Fig. 9

ACS Paragon Plus Environment

SUPPORTING INFORMATION

Design, characterization, and use of a novel amyloid β -protein control for assembly, neurotoxicity, and gene expression studies

Ghiam Yamin^{a, b}, Giovanni Coppola^{b, c}, and David B. Teplow^{b*}

Table S1. Average signal intensity differences among genes after treatment with specific A β assemblies. (Will be available with published version.)

Table S2. Commercially available scrambled A β peptides.

Fig. S1. Phase-contrast microscopy image of primary hippocampal neurons after 7 days in culture. Red scale bar is 91 μ m.

Fig. S2. Relationship between module eigengene (first principal component, corresponding to the weighted summation of expression across all the probes included in a given module) and treatment (A β assembly type). The orange and yellow modules, which are independent from one another, showed the greatest correlation with fibrillar A β 40 and A β 42 treatment among the total of 13 modules defined. Cont: control; S40 and S42: sA β 40 and sA β 42; T40 and T42: LMW A β 40 and LMW A β 42, XL40 and XL42: cross-linked A β 40, cross-linked A β 40 and A β 42; F40 and F42: fibrillar A β 40 and fibrillar A β 42, respectively.

Table S2. Commercially available scrambled A β peptides

Class	Name	Sequence	References
I	Scrambled A β 40	AEGDSHVLKEGAYMEIFDVQGHVFGGKIFRVVDLGSHNVA	1-4
	Scrambled A β 42	AI AEGDSHVLKEGAYMEIFDVQGHVFGGKIFRVVDLGSHNVA	
II	Scrambled A β 40	KVKGLIDGAHIGDLVYEFMDSNSFREGVGAGHVHVAQVEF	5
	Scrambled A β 42	KVKGLIDGAHIGDLVYEFMDSNSAIFREGVGAGHVHVAQVEF	

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Fig. S1

Fig. S2