

Identification of neurotoxic cross-linked amyloid- β dimers in the Alzheimer's brain

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Abstract (words allowed = 120, current = 117)

The primary structure of canonical amyloid β -protein (A β) was elucidated more than 30 years ago, yet the forms of A β which play a role in Alzheimer's disease (AD) pathogenesis remain poorly defined. Studies of AD brain extracts suggest that A β which migrates on SDS-PAGE with a molecular weight of \sim 7 kDa is particularly toxic, however, the nature of this species has been controversial. Using sophisticated mass spectrometry and sensitive assays of disease-relevant toxicity we show that brain-derived bioactive \sim 7 kDa A β comprises a heterogeneous mixture of covalently cross-linked dimers. The identification of A β dimers may open a new phase of Alzheimer research and allow a better understanding of AD, and how to monitor and treat this devastating disorder.

Key words

Amyloid β -protein, long-term potentiation, human neurons, mass spectrometry, video-microscopy.

Introduction

Although the precise cause of Alzheimer's disease (AD) remains obscure, evidence from multiple sources indicate that the amyloid β -protein (A β) plays a key role ([Karran and De Strooper, 2016](#)). There is general agreement that A β monomer is innocuous ([Walsh and Teplow, 2012](#)) or may even have a physiological function ([Pearson and Peers, 2006; Puzzo et al., 2008](#)) and that aggregation and further assembly is required for toxicity ([Yankner and Lu, 2009](#)). Both intrinsic and extrinsic factors may influence how and why wild type A β folds to form toxic assemblies and it is likely that certain post-translational modifications of A β may facilitate formation of toxic structures.

Aqueous extracts of AD brain potently disrupt hippocampal long-term potentiation (LTP), alter synaptic form and number, and impair memory consolidation, and these effects are reversed when A β is depleted by anti-A β antibodies ([Shankar et al., 2008; Barry et al., 2011; Freir et al., 2011; Borlikova et al., 2013; Wang et al., 2017; Hong et al., 2018](#)). The A β in these samples migrate on SDS-PAGE with molecular weights consistent for monomers and dimers ([Shankar et al., 2008; Mc Donald et al., 2010; McDonald et al., 2012; Wang et al., 2017; Yang et al., 2017; Hong et al., 2018; Jin et al., 2018](#)). The ~4 kDa A β species appears to be composed of both native monomers and monomers derived from soluble aggregates that are unstable when electrophoresed in SDS ([Mc Donald et al., 2015](#)). Characterization of the ~7 kDa A β variant from water-soluble extracts of AD brain has been challenging because brain extracts are biologically complex and the amount of ~7 kDa A β is minute. Nonetheless, prior studies that employed non-denaturing size separation and a battery of 12 anti-APP/A β antibodies support the premise that ~7 kDa A β comprises both native A β dimers and dimers generated from the denaturation of

soluble aggregates ([Mc Donald et al., 2015](#)). However, it is not known what sort of bonds hold these dimers together. Moreover, there is continued concern that ~7 kDa A β could be an artefact of SDS-PAGE ([Hepler et al., 2006](#); [Watts et al., 2014](#)).

Here, we isolated the ~4 and ~7 kDa A β species from both the aqueous phase of brain and by solubilizing purified amyloid plaques, and used video microscopy of human neurons and long-term potentiation (LTP) to assess bioactivity. These experiments showed that ~7 kDa A β from both sources potently disrupted neuritic integrity of iPSC-derived neurons and blocked hippocampal LTP, whereas ~4 kDa A β had no effect. Subsequent analysis using mass spectrometry (MS) revealed that ~4 kDa A β contained a rich diversity of A β sequences encompassing a large number of N- and C-termini. Analysis of ~7 kDa A β identified 10 mass matches consistent with covalently cross-linked A β dimers including species such as: 1-37x1-38, 1-38x1-40, 1-40x1-40, 2-40x1-40, 1-40x1-42, and 1-42x1-42. MS-MS of trypsin digested ~7 kDa A β identified the most abundant A β dimer as having a cross-link between Asp1 and Glu22. Collectively, these results recommend the further study of A β dimers as targets for therapy and as potential biomarkers of disease.

Materials and Methods

Reagents and chemicals

A β 1–40 and A β 1–42 were synthesized and purified by Dr. James I. Elliott at the ERI Amyloid laboratory, Oxford, CT, USA. Peptide mass and purity (> 99%) were confirmed by electrospray/ion trap mass spectrometry and reverse-phase HPLC. N-terminally extended (NTE)-A β , -31A β -40, was expressed and purified as described previously ([Szczepankiewicz et al., 2015](#)). Recombinant A η - α (APP505–612) was a gift from Drs. M. Willem and C. Haass (Ludwig-Maximillian University, Munich), and dityrosine cross-linked dimers were produced from recombinant A β (M1-40) ([O'Malley et al., 2014](#)). Peptide standards were prepared as described previously ([Hong et al., 2018](#)) and were stored frozen at 10 ng/ μ l in 50 mM ammonium bicarbonate, pH 8.5.

Gel filtration standards were purchased from Bio-Rad (Hercules, CA). Antibodies and their sources are described in [Table 1](#). All other chemicals and reagents were of the highest purity available and were unless indicated otherwise obtained from Sigma-Aldrich (St. Louis, MO).

Preparation of aqueous extracts from human brain

Frozen brain tissue was provided by the Massachusetts ADRC Neuropathology Core, Massachusetts General Hospital and used in accordance with the Partners Institutional Review Board (Protocol: Walsh BWH 2011). Brain tissue was obtained from [twelve](#) patients with a neuropathological diagnosis of AD ([Table 2](#)). Aqueous extracts were prepared as described previously ([Wang et al., 2017](#)). Ten to 20 grams of cortical gray matter was Dounce-homogenized in 5 volumes of ice-cold artificial cerebrospinal fluid base buffer (aCSF-B) (124 mM NaCl, 2.8 mM

KCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, pH 7.4) supplemented with protease inhibitors (5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethyleneglycoltetraacetic acid, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 2 µg/ml pepstatin, 120 µg/ml pefabloc and 5 mM NaF). The resulting homogenates were centrifuged at 200,000 g for 110 minutes and 4°C in a SW41 Ti rotor (Beckman Coulter, Fullerton, CA) and the upper 80% of the supernatant was removed and dialyzed against fresh aCSF-B, with 3 buffer changes over a 72 hour period. Dialysates were removed to clean tubes, aliquoted and stored at -80°C until required.

Immunoprecipitation/Western Blot detection of Aβ

Half milliliter aliquots of human brain extracts were cleared of antibodies and protein A binding proteins by gently mixing with 15 µL protein A sepharose (PAS) beads for 1 hour at 4°C. PAS beads were removed by centrifugation at 6,000 g for 5 minutes. The supernatant was removed and incubated with 10 µL of AW7 and 15 µL PAS beads overnight at 4°C with gentle agitation. Aβ-antibody-PAS complexes were collected by centrifugation and washed as previously described ([Shankar et al., 2011](#)). Beads were eluted by boiling in 15 µL of 2× sample buffer (50 mM Tris, 2% w/v SDS, 12% v/v glycerol with 0.01% phenol red) and samples electrophoresed on hand-casted 15 well 16% polyacrylamide tris-tricine gels. Proteins were transferred to 0.2 µm nitrocellulose at 400 mA and 4°C for 2 hours. Blots were microwaved in PBS and Aβ detected using anti-Aβ monoclonal antibodies ([Table 1](#)), and bands visualized using a Li-COR Odyssey infrared imaging system (Li-COR, Lincoln, NE). Synthetic Aβ1-40 and Aβ1-42, recombinant NTE-Aβ and Aη-α peptides were loaded to confirm antibody specificity, and to allow comparison between gels.

Isolation of ~4 and ~7 kDa A β using IP/Size Exclusion Chromatography (SEC)

One mL aliquots of aCSF brain extracts were thawed at room temperature, centrifuged at 13,200 g in a bench top centrifuge and the upper 90% of supernatant removed to a clean tube. A total of ten, 1 mL aliquots were IP'd with AW7. Immune-complexes were isolated, washed, and bound A β was eluted by vortexing in 150 μ L 500 mM ammonium hydroxide ([Welzel et al., 2014](#)). PAS beads were removed by centrifugation and eluted a further 2 times with 500 mM ammonium hydroxide, the eluates were pooled and lyophilized. The lyophilizate was resuspended in 1 mL of 88% formic acid (FA) and the entire volume loaded on to a Superdex 75 10/300 GL SEC column. The column was eluted with 50 mM ammonium bicarbonate, pH 8.5 at a flow rate of 0.5 mL/minute and 0.5 mL fractions were collected. Prior to application of sample, the column was calibrated using Blue dextran and gel filtration standards (GFS). The peak fraction containing Blue dextran was designated as fraction zero. Upon collection, fractions were divided into 2 aliquots. One, 50 μ L aliquot was lyophilized and used for WB. The remainder was stored frozen at -80° C. Following WB analysis, fractions which contained ~4 kDa A β and ~7 kDa A β were thawed, pooled, exchanged into neuronal medium and added to iPSCs-derived neurons.

Live-cell imaging of human induced pluripotent stem cells (iPSCs) derived neurons (iNs)

Human iPSCs-derived neurons were induced and cultured as summarized in Supplementary Figure 1A ([Guix et al., 2018](#); [Jin et al., 2018](#)). At iN day 21, neurons were used to investigate the effects of AD brain extracts on neuritic integrity. Approximately 7 hours prior to addition of brain tissue extract sample, images were collected from four fields per well every 2 hours for a total of

6 hours and baseline neurite length and branch points was calculated. During this time, brain extracts were exchanged into neurobasal medium supplemented with B27/Glutamax using PD MidiTrap G-25 columns (GE Healthcare, Milwaukee, WI). Following the 6 hour period of baseline imaging, half of the medium was removed from each well (leaving ~100 µL) and 50 µL of exchanged extract or vehicle, added along with 50 µL of fresh medium. Thereafter, images were collected from four fields per well every 2 hours for at least 72 hours. Phase contrast images sets were analyzed using IncuCyte Zoom 2016A Software (Essen Bioscience, Ann Arbor, MI). The analysis job Neural Track was used to automatically define neurite processes and cell bodies based on phase contrast images. Typical settings were: Segmentation Mode—Brightness; Segmentation Adjustment—1.2; Cell body cluster filter—minimum 500 µm²; Neurite Filtering—Best; Neurite sensitivity—0.4; Neurite Width—2 µm. Total neurite length (in millimeters) and number of branch points were quantified and normalized to the average value measured during the 6 hour period prior to sample addition.

Mouse brain slice preparation and long-term potentiation (LTP) recording

Animal procedures were performed in accordance with the National Institutes of Health Policy on the Use of Animals in Research and were approved by the Harvard Medical School Standing Committee on Animals. Wild type (WT) C57BL/6 mice were purchased from Jackson Labs (Bar Harbor, ME) and a small colony maintained in-house. 2-3 months old mice were anaesthetized with isoflurane and decapitated, then brains were rapidly removed and immediately immersed in ice-cold (0-4°C) artificial cerebrospinal fluid (aCSF). The aCSF contained (in mM): 124 NaCl, 3 KCl, 2.4 CaCl₂, 2 MgSO₄·7H₂O, 1.25 NaH₂PO₄, 26 NaHCO₃ and 10 D-glucose, and was equilibrated

with 95% O₂ and 5% CO₂, pH 7.4, 310 mOsm. Coronal brain slices (350 µm) including hippocampus were prepared using a Leica VT1000 S vibratome (Leica Biosystems Inc, Buffalo Grove, IL) and transferred to an interface chamber and incubated at 34 ± 5°C for 20 minutes and then kept at room temperature for 1 hour before recording.

Brain slices were transferred to a submerged recording chamber and perfused (10 mL/minute) with oxygenated (95% O₂ and 5% CO₂) aCSF for at least 10 minutes prior to electrophysiological recordings. Brain slices were visualized using an infrared and differential interference contrast camera (IR-DIC camera, Hitachi, Japan) mounted on an upright Olympus microscope (Olympus, Tokyo, Japan). Recording electrodes were pulled from borosilicate glass capillaries (Sutter Instruments, Novato, CA) using a micropipette puller (Model P-97; Sutter Instruments, Novato, CA) with resistance ~2 MΩ when filled with aCSF. To induce field excitatory post-synaptic potentials (fEPSPs) in the hippocampal CA1, a tungsten wire stimulating electrode (FHC, Inc., Bowdoin, ME) was placed on the Schaffer collaterals of the CA3 and a recording electrode was placed at least 300 µm away on the striatum radiatum of the CA1. Test stimuli were delivered once every 20 seconds (0.05 Hz) and the stimulus intensity was adjusted to produce a baseline fEPSP of 30–40% of the maximal response of the initial slope of fEPSP. Half mL aliquots of aCSF brain extracts or 40 µL of SEC isolated plaque Aβ were added to an aCSF reservoir to achieve a final volume of 10 ml, and the resulting solutions were perfused over slices for at least 30 minutes. LTP was induced by theta burst stimulation (TBS), composed of 3 trains, each of 4 pulses delivered at 100 Hz, 10 times, with an interburst interval of 200 milliseconds with a 20 second interval between each train. Field potentials were recorded using a Multiclamp amplifier (Multiclamp 700B; Molecular Devices, Sunnyvale, CA) coupled to a Digidata 1440A digitizer. Signal was

sampled at 10 kHz and filtered at 2 kHz and data were analyzed using Clampex 10 software (Molecular Devices, Sunnyvale, CA).

Amyloid plaque isolation and A β purification

Amyloid plaques were isolated from AD brain as described previously ([Selkoe et al., 1986](#)). Whole hemispheres were thawed on ice and 50 to 100 g of cortical gray isolated. Tissue aliquots of ~50 g were incubated with 5 volumes of 2%(w/v) SDS in 50 mM Tris-HCl, pH 7.6, containing 0.1 M β -mercaptoethanol incubated at room temperature for 2 hours. Thereafter, the suspension was Dounce-homogenized and the resultant homogenate was boiled in a water bath for 15 minutes. When samples had cooled to room temperature they were passed through a 112 μ m nylon mesh and the flow-through collected. This material was centrifuged at 300 g for 30 minutes and the pellet collected and washed 3 times with 0.1% SDS in 150 mM NaCl. The final pellet was Dounce-homogenized and then passed through a 38 μ m nylon mesh and the flow-through applied to a preformed sucrose step gradient composed of layers of 1.2 M, 1.4 M, 1.6 M, and 1.8 M sucrose in 1% SDS, 50 mM Tris-HCl, pH 7.6. This preparation was then centrifuged at 71, 840 g for 1 hour at 26°C in a SW28 rotor (Beckman Coulter, Fullerton, CA). Typically, most amyloid plaques are found in the 1.6 M sucrose interface and this material was collected and washed with 5 volumes of 0.1% SDS in 150 mM NaCl. Plaques were then pelleted by centrifugation at 300 g in a bench top centrifuge for 30 minutes. To remove SDS, plaques were washed twice with 1 mL of MilliQ water, and the pelleted plaques were re-suspended in 100 μ L of MilliQ water. A small portion of this material (10 μ L) was stained with 0.2% Congo red and visualized using polarized microscopy.

The remaining plaque suspension was lyophilized and then incubated in 88% FA for 12-14 hour with gentle agitation. Insoluble material was removed by centrifugation at 12,000 g for 15 minutes and the upper 90% of supernatant was used for SEC as described above. Half ml fractions were collected and a small portion (5 µL) was taken for WB analysis. Fractions containing the ~7 kDa A β and ~4 kDa A β monomers were pooled and used for further analysis. For bioactivity experiments approximately equal concentrations (estimated from WBs) of ~4 kDa A β and ~7 kDa A β were diluted directly into aCSF or neuronal medium and used for LTP and live-neuron video microscopy, respectively. For mass spectrometry, samples were lyophilized and shipped to Gothenburg.

Reconstitution of plaque-derived A β

Lyophilized ~4 kDa A β and ~7 kDa A β were reconstituted in 100 µL 1% ammonium hydroxide/20% acetonitrile in water (v/v/v) vortexed briefly and shaken vigorously for 30-60 minutes. The resulting solution was then analyzed using a microflow LC-MS/MS.

In-solution enzymatic digestion of plaque-derived A β

Shortly before mass spectrometric analysis immunoprecipitated (Gunnar, your wrote immunoprecipitated is this correct?) samples were digested with Trypsin/Lys-C Mix (Mass Spec Grade, Promega). Dried eluates were dissolved in 20 µL 50 mM ammonium bicarbonate containing 30 mM dithiothreitol and agitated for 30 minutes at 60 °C. Solutions were then cooled to room temperature and 20 µL 70 mM iodacetamide (in 50 mM ammonium bicarbonate) was

added and the solution agitated in darkness for 30 minutes. Thereafter, 20 µL of 0.08 µg/µL trypsin/Lys-C in 50 mM ammonium bicarbonate was added and gently mixed overnight at 37°C. The entire solution was then transferred to a LC-vial and used for nanoflow LC-MS/MS.

LC-MS/MS

Two different LC setups were used, one configured for flow rates down to 50 µL/min and one configured for flowrates below 1.5 µL/min. The latter configuration was unsuitable for basic pH solvents, while both worked under acidic conditions.

Analysis of intact plaque extracts was performed with basic pH solvents using a microflow LC coupled to electrospray ionization (ESI) orbitrap MS and MS/MS. This was done with a Dionex Ultimate 3000 system (solvent rack SRD-3600, pump NCS-3400RS, autosampler WPS 3000TRS, column oven TCC 3000RS) coupled to a Q Exactive (both Thermo Fisher Scientific) equipped with a HESI-II ion source, using a setup based on a reference measurement procedure for Aβ quantification ([Leinenbach et al., 2014](#)). For analysis with basic mobile phases a reverse-phase monolithic ProSwift RP-4H column (length 25 mm, i.d. 1.0 mm, Thermo Fisher Scientific) was used for separation at a flow rate of 300 µL/min and a linear gradient of 0-50% B for 20 minutes. Mobile phase A consisted of 0.075% ammonium hydroxide/5% acetonitrile in water (v/v/v) while mobile phase B was 0.025% ammonium hydroxide/95% acetonitrile in water (v/v/v).

Mass spectra of intact samples were acquired in positive ion mode with a voltage setting of +4.4 kV and a resolution setting of 70,000 and target values were 1×10^6 both for MS and MS/MS acquisitions. Acquisitions were performed with 1 microscan/acquisition. Precursor isolation

width was 3 *m/z* units. Other setting depended on sample type. For monomer fractions the *m/z* range was 400–2000 to cover possible charge states. Only singly charged ions were deselected for fragmentation. The instrument was operated in data-dependent mode so that each precursor ion scan was followed by 5 fragment ion scans of the 5 most intense ions fragmented by so-called higher energy collision induced dissociation (HCD) at a normalized collision energy (NCE) of 25. Selected *m/z* were excluded for 3 s until eligible again. The ~4 kDa A β monomer fractions from several samples were also analyzed under acidic conditions in the same way as described for the digested samples (see below).

For analysis of the and ~7 kDa A β fractions, our approach was to first observe a meaningful signal, and later to obtain useful fragment acquisitions. Consequently, these samples were analyzed in different ways. The standard *m/z* range setting was 1200–2000 to minimize background. The same fragmentation settings as for monomer fractions normally were used, but also other NCE settings as well as a parallel reaction monitoring approach with every second acquisition set to acquire MS/MS data of the possible 6+ ion of A β 1–42 \times 1–42 with average *m/z* 1502.76.

Analysis of digested samples were performed using acidic pH solvents and a nanoflow LC coupled to ESI orbitrap MS and MS/MS. In this case a different Dionex Ultimate 3000 system (solvent rack SRD-3400, pump NCS-3500RS, autosampler WPS-3000 TPLRS) was coupled to a Q Exactive equipped with a Nanospray Flex ion source. Analysis under acidic conditions was performed with a reversed-phase Acclaim PepMap C18 (length 20 mm, i.d. 75 μ m, particle size 3 μ m, pore size 100 \AA) trap column used for online desalting and sample clean-up, followed by a reverse-phase Acclaim PepMap RSLC C18 (length 150 mm, i.d. 75 μ m, particle size 2 μ m, pore size 100 \AA , both Thermo Fisher Scientific) column. Separation was performed at a flow rate of 300 nL/min by

applying a linear gradient of 0–40% B for 50 minutes. Mobile phase A was 0.1% FA in water (v/v) and mobile phase B was 0.1% FA/84% acetonitrile in water (v/v/v).

Mass spectra of digested samples were acquired in positive ion mode with a voltage setting of +1.7 kV and a resolution setting of 70,000 target values were 1×10^6 both for MS and MS/MS acquisitions. Typical acquisitions were performed with an *m/z* range of 300–1800 with 1 microscan/acquisition. Precursor isolation width was 3 *m/z* units. Singly charged ions with unassigned charged were deselected for fragmentation. The instrument was operated in data-dependent mode so that each MS acquisition was followed by 5 fragment ion acquisitions of the 5 most intense ions, which were fragmented by so-called higher energy collision induced dissociation (HCD) at an NCE of 25. Selected *m/z* were excluded for 5 seconds until eligible again.

Data analysis

Database searches were performed using several different software programs. For spectra from digested samples Proteome Discoverer v2.1 and Peaks Studio v8.5 (Bioinformatics Solutions, Inc., Waterloo, ON, Canada) were used. For spectra from non-digested samples, which required re-determination of the precursor ion monoisotopic *m/z* and charge-deconvolution of the peaks, Mascot Daemon v2.6.0 combined with Mascot Distiller v.2.6.3 (both Matrix Science, London, UK) as well as Peaks Studio v8.5 were used. For Proteome Discoverer and Mascot Deamon/Distiller searches were submitted to the in-house Mascot database server (v2.6.1) while for Peaks Studio the search feature was built-in. Finally the Kojak feature of the Trans Proteomic Pipeline (TPP)

([Keller et al., 2005](#); [Deutsch et al., 2010](#)) was utilized in the search of cross-linked species.

Parameter settings for the software programs are provided in Supplementary Information.

For intact A β mass determinations we also used Xcalibur's built-in deconvolution function, Xtract, and again parameter settings were varied. Typical settings are given in Supplementary Information. Mass lists generated by Xtract and Peaks Studio 8.5 differed slightly. To generate the deconvoluted spectra and peak lists of the intact dimers presented in the figures and tables the two outputs were combined (see Supplementary Information for details).

Data analysis required extensive manual evaluation since we were unable to find a software program that could identify the extensive array of fragment types possible for A β dimers. Therefore, in-house scripts were utilized to produce theoretical fragment lists, which were then compared to obtained acquisitions. The very low background in orbitrap data allows for good signal-to-noise ratios even when the signal is low.

Results

Numerous studies have shown that solubilized amyloid plaques and aqueous extracts of AD brain contain two major A β bands when analyzed by SDS-PAGE ([Masters et al., 1985](#); [Enya et al., 1999](#); [McLean et al., 1999](#); [Morishima-Kawashima et al., 2000](#); [Shankar et al., 2008](#); [Lesne et al., 2013](#); [Watt et al., 2013](#)). The faster migrating species is centered around 4 kDa and the other at ~7 kDa. In pilot studies we reported that ~7 kDa A β isolated from the aqueous phase of a single AD brain possessed AD-relevant toxic activity ([Shankar et al., 2008](#); [Jin et al., 2011](#)). However, the molecular identity of ~7 kDa A β is controversial ([Watt et al., 2013](#); [Willem et al., 2015](#)) and definitive identification has been hampered due to the low abundance of ~7 kDa A β . Here, we used a combination of live-cell imaging, LTP and advanced mass spectrometric techniques to characterize the ~4 and ~7 kDa A β species isolated from the aqueous phase of human brain and from solubilized amyloid plaques.

Bioactive aqueous extracts of AD brains contain A β monomers and ~7 kDa A β .

Initial experiments focused on A β species extracted from the aqueous phase of human brain. In accord with our prior reports ([Shankar et al., 2008](#); [Mc Donald et al., 2010](#); [Mc Donald et al., 2015](#); [Hong et al., 2018](#)), immunoprecipitation/Western blot (IP/WB) analysis revealed two broad bands, one consistent with A β monomer (~4 kDa), and the other a ~7 kDa form of A β ([Figure 1A](#)). When applied to cultured iPSC-derived human neurons (iNs), AD brain extracts caused a time-dependent loss of neuritic complexity ([Figure 1B](#), for analysis of data collected over the last 6 hours of recordings: #1242-mock vs. control, p<0.001; #722-mock vs. control, p<0.001; one-way ANOVA, Supplementary Figure 1). Such extracts also impaired LTP ([Figure 1C](#), statistically

comparison for the last 10 minutes of recording: #722-mock vs. control, p<0.001; #722-mock vs. ID, p<0.001; one-way ANOVA), an electrophysiological correlate of learning and memory ([Bliss et al., 2003](#)). In both cases, depletion of A β from brain extracts prevented these adverse effects ([Figure 1B and C](#)).

Employing the same antibody that was effective in removing bioactivity from brain extracts, we isolated the ~4 and ~7 kDa A β and tested their activity. aCSF extract of AD brain #1242 was IP'd with AW7 and the antibody-bound material eluted with ammonium hydroxide. The eluate was then lyophilized, denatured with FA and the resulting material size-separated using SEC. A portion of each fraction was used for WB'ing. Three 4G8-reactive bands were detected: 1) material that eluted just after the void (fractions 1-3) and migrated at high molecular weight on 16% polyacrylamide SDS gels, 2) SEC low molecular weight species (fractions 9-10) which migrated at ~7 kDa on 16% polyacrylamide SDS gels, and 3) SEC low molecular weight species (fractions 12-14) which migrated at ~4 kDa on 16% polyacrylamide SDS gels ([Figure 1D](#)).

The ~4 and ~7 kDa species are specific for AD brain extracts and are detected by multiple anti-A β antibodies (REF here?). Fractions containing peak ~7 kDa and ~4 kDa species were each pooled and exchanged into iN medium. The amount of 4G8-reactive ~4 and ~7 kDa A β species was quantified by LiCor imaging and equal concentrations (~100 ng/mL) of ~4 and ~7 kDa A β were applied to iN cells and the effects of treatments monitored using video-microscopy. Neurons treated with ~7 kDa A β (red) caused a time-dependent decrease of neurite length, whereas A β monomers (blue) had no effect ([Figure 1E](#)). When neuritic complexity was monitored by quantifying the number of neuritic branch points, again only the ~7 kDa A β (red) had a negative effect (Supplementary Figure 2A).

In parallel experiments, large volumes (15 ml) of aCSF extracts of brain #1185 were IP'd with AW7 and size separated as in Figure 1D. The resulting ~4 and ~7 kDa A β (Supplementary Figure 3A) were then used for LC-MS. Preliminary experiments using a covalently cross-linked dimer and SEC-isolated brain-derived ~4 kDa A β revealed that elution of micro-LC with a weakly alkaline water-acetonitrile gradient ([Leinenbach et al., 2014](#)) allowed better recovery of A β than elution with acidic pH solvents. Application of this approach to SEC-isolated ~4 kDa A β allowed detection of a large array of A β primary structures. For the ~4 kDa fraction of AD brain #1185 (Supplementary Figure 3A) a total of 22 different primary structures were detected (Figure 1F). Similar results were obtained when 3 other aqueous extracts were analyzed ([Gunnar to provide a new Supplementary Figure X?](#) and [Supplementary Table 2](#)), however, when ~7 kDa A β was analysis no assignable peaks were detected.

We hypothesized that the difficulty in detecting ~7 kDa A β by LC-MS could reflect a high level of molecular heterogeneity ([Roberts et al., 2012](#)), i.e. the ~7 kDa band detected by WB may be composed of multiple primary structures of which the amounts of the various components are below the level of quantification by LC-MS. If this were the case, we reasoned that digesting ~7 kDa A β with trypsin/Lys-C would generate common fragments which would be at higher concentrations than their individual precursors. Importantly, when ~7 kDa A β from brain #1185 was digested with trypsin/Lys-C and used for MS/MS a peptide fragment corresponding to A β 17-28 was detected (Figure 1G). These data are consistent with the recognition of ~7 kDa A β by a variety of mid-domain anti-A β antibodies ([Mc Donald et al., 2015](#)) (Figure 2) and provide the first mass spec evidence that ~7 kDa A β contains at least the mid-region sequence of A β . However, other anticipated tryptic fragments (e.g. A β 1-5, 6-16 or 29-40) were not detected.

Aqueous extracts of AD brains and solubilized amyloid plaques contain similar bioactive ~7 kDa A β species.

Since plaques are known to contain ~7 kDa A β , but at much higher quantities than ~7 kDa A β in aqueous AD brain extracts, we sought to systematically compare the properties and activity of ~7 kDa A β from these two sources. Using a well-established method for isolating plaques from human cortex ([Selkoe et al., 1986](#)), we obtained microgram quantities of Congo red-positive plaques ([Figure 2A](#)) from 5 AD brains ([Table 2](#)). Plaques were then solubilized with FA and size-separated using SEC. A portion of each fraction was used for WB'ing and the identified ~7 kDa A β (fractions 8-10, [Figure 2B](#)) and monomer fractions (12-14, [Figure 2B](#)) were each pooled and further analyzed alongside aCSF extracts of AD brain #1849 ([Figure 2C and D](#)).

Plaque-derived material from brains #1242 ([Figure 2B](#)) and #722 ([Supplementary Figure 3B](#)) were mixed with 2x SDS-PAGE sample buffer and electrophoresed alongside AW7 IPs of aCSF extracts of brain #1849 and #453 ([Figure 2C and D](#), and [Supplementary Figure 4](#)) and a battery of 6 different anti-A β antibodies used to identify epitopes present on plaque-derived and aqueous-soluble ~7 kDa A β . Synthetic A β 1-40 and A β 1-42, and recombinant N-terminally extended (NTE)-A β (-31A β 1-42) and A η - α peptides were included to verify the specificity of antibodies and to provide relevant molecular weight markers ([Figure 2D and Supplementary Figure 4B and C](#)). Plaque-derived and aqueous-soluble ~4 kDa bands each resolved into 3 different components, the recognition of which depended on the antibody used ([Figure 2D and Supplementary Figure 4B and C](#)). MAb 3D6 (that needs the 1st amino acid on A β) detected a single band in both sources of monomer, whereas the mid-region antibody, 4G8 and the C-terminal antibodies, 2G3 and

21F12 often detected 2 additional faster migrating bands. Monomer from plaques and aqueous brain showed similar immunoreactivity with the antibodies tested, but the amounts differed. The ~7 kDa A β species from aqueous extracts and plaques also appeared highly similar, and was best recognized by the mid-region (266 and 4G8) and the C-terminal antibodies (2G3 and 21F12) antibodies ([Figure 2D and Supplementary Figure 4B](#)). To investigate whether ~7 kDa A β contained APP sequence N-terminal of A β Asp1, we utilized the anti-APP antibodies 28D10 and 2E9. 28D10 and 2E9 recognize APP sequences 585-600 and 545-555 (695 numbering), respectively. Both antibodies detected recombinant A η - α , but not brain-derived ~4 or ~7 kDa A β , although these species were readily detected when blots were re-probed with 4G8 ([Supplementary Figure 4C](#)).

Having shown that ~7 kDa A β from aqueous extracts and plaques co-migrate on SDS-PAGE, and share several common epitopes, we proceeded to investigate their bioactivity. When applied to iNs at a concentration comparable to that used for aqueous ~7 kDa A β ([Figure 1E](#), ~100 ng/ml) plaque-derived ~7 kDa A β from two different AD brains (#1242 and #722) decreased neurite length by at least 20% ([Figure 2E and Supplementary Figure 3C](#)). Specifically, over the last 6 hours of recording ~7 kDa A β caused a significant decrease in neurite length relative to pre-treatment values, and compared to the time-matched vehicle control ($p<0.001$). In contrast, concentration-matched plaque-derived monomer had no effect ([Figure 2E and Supplementary Figure 3C](#)). Similar results were obtained when branch points were analyzed ([Supplementary Figure 2B](#) and [Supplementary Figure 3D](#)). LTP experiments require much larger sample volumes than used for video-microscopy, therefore we specifically generated large batches of plaque-derived ~4 kDa and ~7 kDa A β ([Supplementary Figure 5](#)) to test on LTP. When used at ~72 ng/ml #1444 plaque-

derived ~7 kDa A β significantly blocked LTP, whereas concentration-matched A β monomer had no effect (~7 kDa A β vs. control, p<0.05; ~4 kDa A β vs. control, p>0.05; one-way ANOVA; Fig. 2F and G). Collectively, these results demonstrate that plaque-derived ~7 kDa A β and aqueous ~7 kDa A β have similar biochemical, immunological, and toxic properties, thus indicating that their molecular structure is the same, or highly similar.

LC-MS indicates that solubilized amyloid plaques contain both covalently cross-linked A β heterodimers and a diverse mixture of A β monomers.

Since it had been relatively straight forward to obtain mass estimates for intact aqueous ~4 kDa A β using LC-MS/MS and mildly alkaline solvents (Figure 1F), we began our analysis of plaque-derived A β using the same approach. MS/MS analysis of SEC-isolated ~4 kDa A β from five different brains identified a total of 36 A β primary structures (Figure 3A and Figure X1). Many peptides were detected in all brains, but there were notable differences between samples. Specifically, there was clear segregation between brains in which the predominant peptides terminated at Ala42 versus Val40. In 3 brains (#1185, #722 and #1167) most peptides terminated at Ala42, whereas in the other 2 brains (#1242 and #464) most peptides terminated at Val40. In these latter 2 brains, peptides terminating at Val39, Gly38 and Gly37 were also evident. These differences in Ala42/Val40 content are also reflected in WB analysis of brains #1242 and #722 (Figure 2 and Supplementary Figure 4). MS/MS identified A β peptides with 14 different N-termini, and many of the detected peptides, except the previously elusive p3 fragment (A β 17-42), had been reported previously ([Mori et al., 1992](#); [Miller et al., 1993](#); [Portelius et al., 2010](#)). In addition, oxidized peptides as well as occasional shorter peptides were also detected ([SI Table X1](#)).

Consistent with our analysis of aqueous ~7 kDa A β (Figure 1), MS analysis of plaque-derived ~7 kDa A β proved challenging. In a veritable forest of isotopically resolved peaks it was nevertheless possible to distinguish features matching potential A β dimers, particularly A β 1–42 \times 1–42 (Figure 3B). Interestingly, in brains were A β 40 was the dominant monomer (#464 and #1242), A β 1–40 \times 1–40 was the most prominent ~7 kDa A β .

By setting the instrument to acquire MS/MS data from *m/z* 1502.76 during the whole acquisition it was possible to obtain useful data from this peak. (Gunnar, please add a sentence to explain why 1502,76 was used.) The fragment mass spectrum obtained from brain #1185 ~7 kDa A β clearly detected A β 1–42 \times 1–42 (SI Fig. X2), and analysis of brain #1242 also yielded clear-cut data. Fig. 3B shows an average trace over the whole *m/z* range and extracted MS/MS spectra. Dimers composed of monomers terminating at Val40 were the most abundant species, but dimers containing A β terminating at Gly37, Gly38, Val39 and Ala42 are also detected (Figure 3B). Excluding oxidized species, ten different variants were confirmed, seven of which may occur in two orientations. The most abundant dimer identified was A β 1–40 \times 1–40. The fragment pattern from these dimers are very similar to that of monomeric A β 1–40/42 (ref?) with long b-ions dominating the fragment ion spectrum (Supplementary Figures X2-X4), but only one of the two A β chains appeared to fragment. For A β 1–40 \times 1–40 only one ladder of long b-ions is formed (SI Fig. X3) and for A β 1–38 \times 1–40 two such ladders can be seen (SI Fig. X4). This is quite different from the pattern seen with synthetically produced [A β (M1–40)]DiY dimer, where multiple ladders were observed showing a fragmentation pattern involving both chains (SI Fig. X5). Collectively, these data indicate that our samples did not contain appreciable levels of the

dityrosine dimers which have been suggested to be present in brain extracts ([Vazquez de la Torre et al., 2018](#)).

Having proven that ~7 kDa A β is a covalently linked A β dimer, we were anxious to determine the position(s) at which the component monomers are linked. Given the highly heterogeneous primary structures evident in MS/MS spectra for ~7 kDa (Figure 3B), we investigated whether proteolytic digestion might be useful to enrich common peptide fragments that contain cross-linked amino acids.

LC-MS/MS analysis of digested ~7 kDa A β identifies a covalent link between Asp1 and Glu22

When plaque-derived ~7 kDa A β from brain #1242 was subjected to digestion with trypsin/Lys-C the dimer-specific fragment, A β 17–28 \times 1–5, was readily detected (Figure 4A and Supplementary Figure X6). Importantly, this fragment was found in multiple different digestion experiments using ammonium bicarbonate, and in separate digestions done in ammonium hydroxide. Trypsin/Lys-C, cleaves after Lys and Arg residues and the deconvoluted fragment spectrum shown in Figure 4A (and in schematic form in Figure 4B) provide compelling evidence that ~7 kDa A β is composed of 2 A β chains linked between Glu22 and Asp1. TPP/Kojak analysis corroborates this conclusion. **Distiller? -Gunnar I'm not sure what you want to say?** However, in view of the facts that: (1) we did not observe the A β 17–28 \times 1–5 fragment in samples that contained a verifiable intact dimer (e.g. #1185), and (2) ~7 kDa A β is highly heterogeneous, it is seems likely that dimers may arise due to linkages at other sites, besides the Glu22xAsp1 link we have confirmed. **Gunnar, do you want to expand?**

Discussion

Prior work from our lab and others indicated that human brain extracts which contain SDS-stable ~7 kDa A β exert a range of AD-relevant pathophysiological effects ([Shankar et al., 2008](#); [Freir et al., 2011](#); [Jin et al., 2011](#); [Larson et al., 2012](#); [Borlikova et al., 2013](#); [Yang et al., 2017](#); [Hong et al., 2018](#)). In a pilot study, we found that native ~7 kDa A β size-isolated from the aqueous phase of a single AD brain blocked LTP ([Shankar et al., 2008](#)), and in a separate study, that immunopurified aqueous ~7 kDa A β induced aberrant phosphorylation of tau and neuritic degeneration ([Jin et al., 2011](#)). Yet, until now the molecular composition of bioactive ~7 kDa A β was unknown.

Here, our initial efforts focused on analysis of the ~4 and ~7 kDa A β species that had been immunoisolated and size-separated from bioactive aqueous AD brain extracts. LC-MS readily identified an array of primary structures in the A β monomer fraction, but failed to identify any interpretable signal in the ~7 kDa A β fraction. Given our demonstration that ~4 kDa A β contained at least 36 different primary structures (not counting oxidized variants) it is evident that a very large number of molecularly distinct dimers could be formed ([Roberts et al., 2012](#)), and that this may explain why it was not possible to detect a meaningful signal for ~7 kDa A β . Following this logic, we reasoned that proteolytic digestion of ~7 kDa A β would generate common fragments at concentrations approaching the sum of their individual precursors. Trypsin/Lys-C cleaves C-terminal of Lys and Arg residues, such that digestion of A β 1-40, is predicted to generate four fragments: 1-5; 6-16; 17-28; and 29-40. However, cleavage of A β sequences with variable N- and C-termini will give rise to fragments with variable N- and C-termini, whereas the internal 17-28 fragment should be common to all A β species and therefore should be the most readily detected. When ~7 kDa A β was digested with trypsin/Lys-C a peptide fragment corresponding to A β 17-28

was indeed detected, but no other fragments were found. These data confirm that ~7 kDa A β contains at least the mid-region of A β , and are consistent with ~7 kDa A β having heterogeneous N- and C-termini, but no obvious covalent cross-links involving residues 17-28.

The inability to identify the expected next most abundant fragment, 6-16, indicated that we were working close to the limit of reliable detection and that much higher concentrations of ~7 kDa A β would be required for full elucidation of this material. Since ~7 kDa A β is present in aqueous extracts at less than 200 ng per gram of wet weight gray matter ([Mc Donald et al., 2010](#); [McDonald et al., 2012](#); [Mc Donald et al., 2015](#)) we turned to another more concentrated source of ~7 kDa A β – amyloid plaques ([Masters et al., 1985](#); [Roher et al., 1996](#); [Sergeant et al., 2003](#); [Shankar et al., 2008](#)). Before pursuing the identification of plaque-derived ~7 kDa A β we directly compared the biochemical, immunological, and bioactive properties of this material, versus aqueous ~7 kDa A β . By every parameter tested ~7 kDa A β from these two sources behaved highly similarly. Therefore, we determined that elucidating the structure of plaque-derived ~7 kDa A β would provide insight about aqueous ~7 kDa A β .

LC-MS analysis of plaque-derived ~7 kDa A β from 5 different AD brains unequivocally demonstrated the presence of a family of covalently cross-linked A β dimers that included: 1-42x1-42, 1-40x1-40 and 1-40x1-38. However, because of the complexity of the spectra and the potential for confounding post-translational modifications it was not possible to unambiguously identify the sites at which the component monomers were cross-linked. Thus, we again turned to tryptic digestion to simplify the study of these heterogeneous samples. Careful analysis of MS-MS spectra identified a branched fragment in which amino acids 17-28 were linked to residues 1-5 at Glu22 and Asp1. Given that the intact mass of dimers was 18 mass units less than the sum

of the mass of 2 component monomers, it appears that the most common point of linkage arises from a condensation reaction between the alpha nitrogen of Asp1 and the carboxylic acid group of Glu22. Such cross links are uncommon, but certain conformations in which a H-bond donor or Lewis acid group are positioned near the carboxylate group may favor their formation. It is noteworthy that in certain plaque-derived ~7 kDa A β samples MS analysis of undigested samples confirmed the presence of covalent A β dimers, but several of these did not yield a detectable signal for the 17-28x1-5 fragment. These results imply that ~7 kDa A β is heterogeneous both with regard to the component monomers which contribute to dimers and the linkages which hold dimers together. Indeed, it has not escaped our attention that aqueous ~7 kDa A β did not yield a 17-28x1-5 tryptic fragment.

Other possible cross-links consistent with our results from undigested ~7 kDa A β could arise from condensation reactions involving alternate amino terminal residues besides Asp1 and the involvement of carboxylates from amino acids other than Glu22. In addition, *in vitro* studies have shown that A β monomers can be induced to form covalent dimers by the phenolic coupling of tyrosine residues ([Galeazzi et al., 1999](#); [Al-Hilaly et al., 2013](#); [O'Malley et al., 2014](#); [O'Malley et al., 2016](#); [Vazquez de la Torre et al., 2018](#)) and the action of the enzyme transglutaminase which can catalyze the formation of an isopeptide bond between Gln15 and Lys16 ([Hartley et al., 2008](#); [O'Malley et al., 2016](#)). Importantly, in AD there is evidence of increased protein cross-linking involving both oxidative phenolic coupling ([Hensley et al., 1998](#); [Al-Hilaly et al., 2013](#)) and transglutaminase-mediated isopeptide formation ([Wilhelmus et al., 2009](#)). However, MS of digested ~7 kDa A β did not identify the 6-16x6-16 fragments that should be derived from diytrosine or isopeptide cross-linked A β species. Of course, the absence of proof is not the proof

of absence, and further investigations using a larger collection of brains will be required to define the array of cross-links that facilitate A β dimer formation.

Prior studies that employed synthetic A β cross-linked at different sites suggest that different dimers may share some properties, but also exhibit important differences (reviewed in ([Vazquez de la Torre et al., 2018](#))). For instance, dimers tend to aggregate more rapidly and form more intermediate-sized aggregates than monomers, but the type of aggregates formed are quite different ([O'Malley et al., 2016](#)). How these properties effect bioactivity has not been thoroughly investigated and whether all molecularly distinct dimers, or only a sub-population are toxic will require further study.

Our unambiguous demonstration that covalent dimers are present in human brain and that at least some fraction of this material has disease-relevant bioactivity opens up a completely new area of AD research. For instance, while there have been some suggestions that the levels of dimers, or antibodies that recognize dimers are elevated in AD CSF and blood ([Moir et al., 2005](#); [Klyubin et al., 2008](#); [Villemagne et al., 2010](#)), measurement of A β dimers in human biofluids has not been systematically investigated. Clearly it will be important to generate dimer-specific antibodies with a preference for those dimers proven to be toxic. Similarly, while recent results from anti-A β immunotherapy trials have been encouraging ([Logovinsky et al., 2016](#); [Satlin et al., 2016](#); [Sevigny et al., 2016](#)) it will be important to assess whether test antibodies can engage with toxic A β dimers. Moreover, effort should be devoted to understand the process(es) by which dimers are formed and whether these can be modulated.

Acknowledgments

We thank Dr. Tracy Young-Pearse for the gift of virally infected iPSCs, and Drs. Frederique Bard and Christian Haass for providing antibodies. We thank Dr. Jessica Mc Donald for conducting preliminary experiments and Ms. Ali Desousa for helping establish the plaque purification procedure. This work was supported by grants to DMW from Science Foundation Ireland (Grant 08/1N.1/B2033), the National Institute on Aging (AG046275), Bright Focus, the Swedish Alzheimer Foundation, the Swedish Brain Foundation, the Swedish Research Council and the European Research Council. HZ is a Wallenberg Academy Fellow. KB holds the Torsten Söderberg Professorship at the Royal Swedish Academy of Sciences.

Gunnar, Kaj and Henrik to add acknowledgments.

Disclosures

None of the authors have biomedical financial interests or potential conflicts of interest related to the work performed in the present study. Unrelated to the current study, KB has served as a consultant or at advisory boards for Alzheon, BioArctic, Biogen, Eli Lilly, Fujirebio Europe, IBL International, Merck, Novartis, Pfizer, and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. Unrelated to this study, HZ has served at scientific advisory boards for Eli Lilly, Roche Diagnostics, Wave, Samumed and CogRx, has received travel support from Teva and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg.

Author Attributions

DMW conceived the project. DMW, HZ and KB designed and supervised the research. BG performed, designed and interpreted all mass spec experiments. EP assisted with the design and interpretation of certain mass spec experiments. TTO'M produced the [A β (M1–40)]DiY dimer and with WH purified aqueous A β species. WH, WL and XS purified and analyzed A β species from plaques. DJS provided guidance on plaque purification. WH and WL conducted all video-microscopy experiments. ZW conducted all electrophysiology experiments and MPF performed postmortem analyses and assessed the disease status of patient brains. DMW, GB and WH wrote the manuscript. All the authors critically assessed and contributed to writing the manuscript.

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Figure legends

Figure 1 Bioactive aqueous extracts of AD brains contain A β monomers and ~7 kDa A β species.

(A) aCSF extracts of AD brains (#722, #414 and #1242) contain A β species that migrate on SDS-PAGE at ~4 kDa (single arrow) and ~7 kDa (double arrow). Samples were immunoprecipitated (IP'd) with the pan anti-A β antiserum, AW7, or pre-immune serum (PI) and blotted with the mid-region anti-A β mAb, 4G8. Non-specific bands detected when PI was used are indicated on the right, and migration of molecular weight marker is on the left. (B) Time-course plots show that A β -containing aCSF extracts of brains #1242 (red open squares) and #722 (red filled circle) cause neuritotoxicity compared to the medium alone control (black) (last 6 hours; #1242-mock vs. control, p<0.001; #722-mock vs. control, p<0.001; one-way ANOVA), or brain extracts immunodepleted (ID) of A β with AW7; #1242-ID (blue open squares) and #722-ID (blue filled circles) (last 6 hours; #1242-mock vs. ID, p<0.001; #722-mock vs. ID, p<0.001; one-way ANOVA).

(C) The A β -containing aCSF extract of brain #722 (red) blocks LTP compared to aCSF vehicle control (black) or #722-ID (blue) (last 10 minutes; #722-mock vs. control, p<0.001; #722-mock vs. ID, p<0.001; one-way ANOVA). (D) aCSF extract of brain #1242 was IP'd with S97-beads, antigen eluted with 500 mM ammonium hydroxide, the eluate lyophilized, the lyophilizate dissolved in FA and the resulting solution subjected to SEC, and fractions used for WB with 4G8. (E) The A β monomer and ~7 kDa A β fractions shown in D were exchanged into iN culture medium and added to iN cells. ~7 kDa A β , but not A β monomer, caused time-dependent neuritotoxicity (last 6 hours; ~7 kDa A β vs. control, p<0.001; ~4 kDa A β vs. control, p>0.05; one-way ANOVA). (F) Liquid chromatography (LC)-mass spectrometry (MS) analysis of SEC fractions containing the ~4 kDa A β species detected 22 different A β primary structures. Data are shown as relative abundance of

detected peaks, with the most abundant peak ($\text{A}\beta$ 1-42) set to a value of 1. **(G)** MS/MS analysis of trypsin-digested SEC fractions containing the ~7 kDa species confirmed the presence of $\text{A}\beta$ residues 17-28.

Figure 2 Aqueous extracts of AD brains and solubilized amyloid plaques contain similar bioactive ~7 kDa $\text{A}\beta$ species. **(A)** Congo red positive amyloid plaques were isolated from AD brain #1242, and **(B)** dissolved in formic acid, chromatographed on SEC and used for WB with 4G8. The elution of Blue dextran (BD) and globular protein standards is indicated by downward point arrows and SDS-PAGE molecular weight standards are on the left. **(C-D)** Plaque SEC-isolated ~4 and ~7 kDa fractions, and AW7 IPs of aCSF extracts were used for WB'ing employing 6 different anti- $\text{A}\beta$ mAbs. Synthetic $\text{A}\beta$ 1-40, $\text{A}\beta$ 1-42 and recombinant NTE- $\text{A}\beta$ (-31 $\text{A}\beta$ 1-42) and $\text{A}\eta\text{-}\alpha$ peptides were loaded as controls. Single arrow and double arrows indicate the ~4 and ~7 kDa $\text{A}\beta$ species, respectively, and an asterisk marks the position of $\text{A}\eta\text{-}\alpha$. The ~7kDa $\text{A}\beta$ species from aCSF extracts and solubilized plaques co-migrate and exhibit similar immunoreactivity anti- $\text{A}\beta$ mAbs. **(E)** Time-course plots show that 1242 plaque-derived ~7 kDa $\text{A}\beta$ species (red) causes neuritotoxicity, whereas 1242 plaque-derived $\text{A}\beta$ monomer (blue) is indistinguishable from the medium alone control (black) (last 6 hours; ~7 kDa $\text{A}\beta$ vs. control, $p<0.001$; ~4 kDa $\text{A}\beta$ vs. control, $p>0.05$; one-way ANOVA). **(F-G)** Time-course plots show that 1444 plaque-derived ~7 kDa $\text{A}\beta$ (red), but not $\text{A}\beta$ monomer (blue), blocks LTP compared to aCSF control (black) (last 10 minutes; ~7 kDa $\text{A}\beta$ vs. control, $p<0.05$; ~4 kDa $\text{A}\beta$ vs. control, $p>0.05$; one-way ANOVA).

Figure 3 Solubilized amyloid plaques contain covalently cross-linked A β heterodimers and a diverse mixture of A β monomers. (A) LC-MS detects a rich array of A β primary structures in SEC-isolated monomer fractions of solubilized plaque. Results are for monomers isolated from 5 brains (#1185, #722, #1167, #1242 and #464). The height of cones corresponds to the relative abundance of detected peaks, with the most abundant peak in each sample set to a value of 1. The identity of the peaks shown were confirmed by tandem MS/MS (see Supplementary Table X1). (B) LC-MS of the ~7 kDa fractions from brain #1242 reveals the presence of A β species consistent with covalently cross-linked heterodimers. The upper panel shows the average data for the retention time span 9-11 min and the magnified panel shows five 0.25 min retention time spans. The right panel illustrates structures of A β dimers consistent with the identified masses.

Figure 4 A β heterodimers are covalently cross-linked and the most abundant species is linked between Asp1 and Glu22. (A) Deconvoluted fragment ion spectrum obtained from tandem MS/MS of the tryptic peptide A β 17-28x1-5 from the ~7 kDa plaque-derived fraction of brain #1242 shows peaks from several different fragment ion types. Peaks corresponding to identified A β sequence are colored and unassigned peaks are in grey. (B) Schematic of tryptic peptides released from a putative A β 1-40-A β 1-40 dimer crosslinked at D1 and E22, and A β 17-28x1-5 related fragments detected in (A).

Supplementary Figure 1. Treatment of iNs with aqueous AD brain extracts induces neuritic dystrophy. (A) Schematic depicts the process used to generate mature iNs and the time interval for sample addition and live cell imaging. (B) On iN day 21, cells were treated with medium alone (control) or brain extracts (#1242-mock and #1242-ID) and cells imaged for 72 hours. Phase contrast images (top panel) were used to identify neurites (middle panel) using the IncuCyte NeuroTrack analysis. Identified neurites (purple) are shown on the phase contrast images (bottom panel). Scale bars are 100 μ m. (C) Time-course plots show that A β -containing aCSF extracts of brains #1242 (red filled circle) and #722 (blue filled circle) cause neuritotoxicity compared to the medium alone control (black) (comparison over last 6 hours of recording: #1242-mock vs. control, p<0.001; #722-mock vs. control, p<0.001; one-way ANOVA), or brain extracts immunodepleted (ID) of A β with AW7; #1242-ID (red open circle) and #722-ID (blue open circle) (comparison over last 6 hours of recording: #1242-mock vs. ID, p<0.001; #722-mock vs. ID, p<0.001; one-way ANOVA). Each well of iNs was imaged for 6 h prior to addition of sample and NeuroTrack-identified neurite branch points used to normalize neurite branch points measured at each interval after addition of sample. Cells treated with medium alone were used to monitor the integrity of untreated cells.

Supplementary Figure 2 Both aqueous ~7 kDa A β and plaque-derived ~7 kDa A β are neuritotoxic. The effect of ~7 kDa A β from brain #1242 on neuritic branch points was quantified using the same neurons shown in Figure 1E and Figure 2E. (A) Time-course plots show that the aqueous ~7 kDa A β , but not A β monomer, caused time-dependent neuritotoxicity (comparison over last 6 hours of recording: ~7 kDa A β vs. control, p<0.01; ~4 kDa A β vs. control, p>0.05; one-

way ANOVA). **(B)** Branch points of the A β monomer and ~7 kDa A β treated iNs as shown in Figure 2E. Time-course lots show that the ~7 kDa A β from AD amyloid plaques, but not A β monomer, caused time-dependent neuritotoxicity (comparison over last 6 hours of recording: ~7 kDa A β vs. control, p<0.001; ~4 kDa A β vs. control, p>0.05; one-way ANOVA).

Supplementary Figure 3 Plaque-derived ~7 kDa A β species from AD brain #722 is neuritotoxic.

(A) aCSF extract of brain #1185 was IP'd with AW7-beads and used for SEC as described in the Methods, and fractions analyzed by WB with 2G3 and 21F12. The fractions which contained ~7

kDa A β and monomers were used for the mass spectrometric analysis shown in Figure 1F and G.

(B) Amyloid plaques isolated from AD brains #722 were dissolved in formic acid, chromatographed on SEC and analyzed by WB with 2G3 and 21F12. **(C and D)** Equal amount of

the A β monomer and ~7 kDa A β fractions shown in **B** were applied to iNs. Time-course plots of

relative neurite length (**C**) and branch points (**D**) show that plaque-derived ~7 kDa A β species (red)

cause neuritotoxicity, whereas plaque-derived A β monomer (blue) does not (comparison over

last 6 hours of recording: ~7 kDa A β vs. control, p<0.001; ~4 kDa A β vs. control, p>0.05; one-way

ANOVA). The medium vehicle control is shown in black.

Supplementary Figure 4 The ~7 kDa A β species isolated from plaques co-migrate with the ~7

kDa A β in aCSF brain extracts. (A) A total of 8 mAbs were used to investigate the primary

structure of plaque-derived and aCSF extracted A β . **(B)** Plaque SEC-isolated ~4 and ~7 kDa

fractions, and AW7 IPs of aCSF extracts were used for WB'ing employing 6 different anti-A β mAbs,

and **(C)** 2 mAbs with epitopes in APP N-terminal of A β . The ~7 kDa A β species from aCSF extracts

and solubilized plaques co-migrate and react similarly with anti-A β mAbs, but are not recognized by the anti-APP mAbs 2E9 or 28D10. **(C)** Re-probing the 28D10 blot with 4G8 reveals ~4 and ~7 kDa A β species are present. Synthetic A β 1-40, A β 1-42, recombinant N-terminally extended (NTE)-A β (-31A β 1-42) and recombinant A η - α were loaded as controls. A single arrow indicates A β monomer, double arrows ~7 kDa A β , and an asterisk denotes A η - α .

Supplementary Figure 5 Western blot analysis of plaques isolated from brain #1444. Amyloid plaques from AD brain #1444 were dissolved in formic acid, chromatographed on SEC and analyzed by WB with 2G3 and 21F12. The elution of Blue dextran (BD) and globular protein standards is indicated by downward point arrows and SDS-PAGE molecular weight standards are on the left. Single arrow and double arrows indicate the ~4 and ~7 kDa A β , respectively. Synthetic A β 1-42 loading controls are in the last 3 lanes. Fractions (8-10) containing ~4 kDa A β and fractions (13-15) containing ~7 kDa A β were pooled and used for LTP experiments shown in Figure 2F and G.

Supplementary Figure X1. LC-MS identifies a rich array of plaque-derived monomer primary structures both within and between brains. Spectra are shown for 4 LC retention time spans for plaque-derived SEC-isolated monomer fractions of brains #1185, #722, #1167, #1242 and #464. The x-axis indicates m/z values between 850-1330, the y-axis signal intensity, and the z-axis the HPLC retention time span. The identity of the peaks shown were confirmed by MS/MS (see SI Table X1).

Supplementary Figure X2. Deconvoluted fragment ion spectrum of the plaque-derived intact dimer A β 1-42 \times 1-42 from brain #1185. The schematic shows an A β 1-42 homo-dimer cross-linked between D1 and E22. The α -chain is shown in green and the β -chain in orange, and all potential fragmentation sites are indicated. The actual spectrum for plaque-derived intact ~7 kDa A β from brain #1185 is shown in 3 separate panels, [M+H] $^+$: 0-2,500 (upper panel), 2,500-6,500 (middle panel), and 6,500-9,000 (lower panel). Peaks from three different fragment ion series are indicated together with the respective sequence ladders; α -chain b-ions (green), β -chain y-ions (italic orange), and α (1-42) \times β (1-42)-chain b-ions (blue). Unassigned peaks are shown in light grey. Only one ladder is observed, indicating that for an individual dimer ion only one of the chains is subject to fragmentation (cf. SI Fig. X5).

Supplementary Figure X3. Deconvoluted fragment ion spectrum of the of the plaque-derived intact dimer A β 1-40 \times 1-40 from brain 1242. The schematic shows an A β 1-40 homo-dimer cross-linked between D1 and E22. The α -chain is shown in green and the β -chain in orange, and fragmentation sites corresponding to detected fragments are indicated. The actual spectrum for plaque-derived intact ~7 kDa A β from brain #1242 is shown in 3 separate panels, [M+H] $^+$: 0-2,500 (upper panel), 2,500-6,500 (middle panel), and 6,500-8,???? (lower panel). Peaks from three different fragment ion series indicated together with the respective sequence ladders; α -chain b-ions (green), β -chain y-ions (italic orange), and α (1-40) \times β (1-40)-chain b-ions (blue). Unassigned peaks are in light grey. Only one ladder is observed, indicating that for an individual dimer ion

only one of the chains is subject to fragmentation (cf. SI Fig. X5). However, peaks corresponding to cleavages between E22 and D1 must originate from the β -chain.

Supplementary Figure X4. Deconvoluted fragment ion spectrum of the intact A β 1-40 \times 1-38 and A β 1-38 \times 1-40 heterodimer from brain 1242. The schematic shows two possible A β 1-40 \times A β 1-38 hetero-dimers cross-linked between D1 and E22. The α -chain is shown in green for 1-40 on the left panel, and 1-38 on the right panel. The β -chain is in blue for 1-38 on the right panel, and in orange for 1-40 of the left panel. Fragmentation sites corresponding to detected fragments are indicated. The actual spectrum for plaque-derived intact ~7 kDa A β from brain #1242 is shown in 3 panels with $[M+H]^+$ ranging from: 0-2,500 (upper panel), 2,500-6,500 (middle panel), and 6,500-8,???? (lower panel). Peaks from five different fragment ion series are indicated with the respective sequence ladders; α -chain b-ions (green), β (1-38)-chain y-ions (italic blue), β (1-40)-chain y-ions (italic orange), α (1-40) \times β (1-38)-chain b-ions (blue), and α (1-38) \times β (1-40)-chain b-ions (orange). Unassigned peaks are in light grey. Two ladders are observed (one for each dimer type) indicating that only one chain (presumably the β -chain) from each dimer type is subject to fragmentation.

Supplementary Figure X5. Deconvoluted fragment ion spectrum of recombinant dityrosine dimer. Dimers were formed by enzymatic oxidation of recombinant A β (M1-40) to generate 1-40 homodimers linked between Y10 of one monomer chain and the Y10 of the apposing monomer and an expected mass of 8,921. The spectrum spanning the $[M+H]^+$ from 7,100 to 9,100 is shown.

Fragment ions corresponding to simultaneous fragmentation of both the α - and β -chains is evident, but since the α - and β -chains are equivalent, only the longest α -chain are annotations.

Supplementary Figure X6. Detailed fragment ion spectrum of the tryptic peptide A β 17-28 \times 1-5 from the ~7 kDa plaque-derived fraction of brain #1242. (A) Deconvoluted fragment ion spectrum from tandem MS/MS of the tryptic peptide A β 17-28 \times 1-5 with all identified peaks indicated both with sequence and using the nomenclature of Ref. [reference] extended with internal fragments. Peaks corresponding to identified A β sequence are colored and unassigned peaks are in grey. (B) Schematic of the tryptic peptides A β 17-28 \times 1-5 detected in the ~7 kDa plaque-derived fraction isolated from brain 1242. A β heterodimers are covalently cross-linked and the most abundant species is linked at D1 and E22.

Supplementary Information

MS and MS/MS acquisition settings

Both LC systems were connected through Thermo Scientific SII for Xcalibur 1.3.0.73 with Chromeleon 7.2.4.8179 for LC control. The acquisition software used was Thermo Foundation 3.1.64.11 with Xcalibur 3.1.66.10 and instrument configuration Q Exactive - Orbitrap MS 2.8 SP1 build 2806.

Comments on acquisition settings and data analysis

The instrument settings commonly used for proteolytic peptides frequently result in failure to acquire MS/MS spectra for larger peptides even though the signal intensity is well above the require threshold. Generally it is desirable to enable the “Peptide match” selection, which would assign the monoisotopic peak m/z of the precursor to the fragment io acquisition. This way the precursor m/z does not require correction prior to database search. With larger peptides this feature could not be enabled and therefore more specialized processing program is required, such as Mascot Distiller or PEAKS Studio. Use of these programs are also desirable for other reasons as well. For example, Mascot takes into account only singly and doubly charged fragment ions, and for precursors with higher charge state than 3, fragment ions with charge states >2 are produced. To include these, the software must deconvolute multiply charge ions into singly charged ion (or non-charged species).

We were unable to access a software that did not require prior knowledge of the mass of the link. A program allowing a liberal mass tolerance setting would have been useful; unfortunately a large tolerance of tens or hundreds of Da is typically not allowed. Therefore, the search strategy was to construct a large amount of different PTMs consisting of possible combinations of peptides and links. In the particular case with dimers, PEAKS Studio 8.5 worked well enough since it was possible to perform searches with a large amount of modifications. To speed up the processing we constructed 2 small databases, one containing only APP isoforms and one containing only A β 1-43.

Once the nature of the link was established, both Mascot Distiller and Peaks Studio were able to process both intact and tryptic dimer data; Peaks Studio, however, provided a smoother workflow. We also took advantage of the Xcalibur's built-in Xtract function to charge and isotope deconvolute spectra. The deconvoluted peak lists produced by Peaks Studio and Xtract differed to some extent and we combined them to obtain more complete fragment ion lists for the intact dimers. The lists were also validated manually against the unprocessed fragment ion acquisition to ensure that the data produced by the algorithms were trustworthy.

Spectra from digested samples were also subjected to analysis by Kojak/TPP. This software allows analysis of linked peptides. Also here the link mass has to be known beforehand, which limits the usefulness when the objective is to screen for peptides with an endogenous, unknown link. Nevertheless, once the number of linking possibilities had been narrowed down to water and ammonia loss Kojak/TPP was a handy tool to confirm findings and also suggest other linked peptide variants.

Hitherto, we have not found a really smooth workflow but are still relying on a combination of software with some manual data handling in between. By using in-house developed scripts simpler data extraction can be semi-automated, which reduces analysis time significantly.

Mascot Daemon search parameters – search using Mascot Distiller for spectrum processing

All searches [Database = custom made Abeta1-43, APP human isoforms, Uniprot_SwissProt; Fixed modifications = none; Variable modifications = Oxidation (M), Glu→pyro-Glu (N-term E), and various “dimer PTMs”; Decoy = not enabled; Enzyme = none; Max. missed cleavages = not relevant; Monoisotopic = selected; Peptide charge = 1+; Peptide tol. = 20 ppm (digested samples)/1.2 Da (intact samples); #13C = 0].

MS/MS [MS/MS ion search = enabled; Error tolerant search = not enabled; Data format = Mascot generic; MS/MS tolerance = 50 mmu (digested samples)/1.2 Da (intact samples); Quantitation = none; instrument type = CID fragment type spectra – all singly charged *b*- and *y*-ions].

Data import filter = Mascot Distiller [Data File Format = ThermoXcalibur; Default for unknown scan type = Profile/continuum; Data import filter options: Mascot Distiller Processing Options = see below; Multi-Sample Files = Separate search for each sample; Peak List Format = MGF; Intensity values = Area; Scan Range (multi-scan files) = sample-dependent; Distiller Project File Save = enabled; Output PMF Masses as = MH+; Output MS/MS Fragments as = MH+].

Mascot Distiller Processing Options

MS Processing: Un-centroiding [Peak half width = 0.025; Data points per Da = 100; Data points per Da = 100; Always uncentroid = enabled]; Re-gridding [Data points per Da = 100]; Multi-Format Spectrum [Preferred type = Profile]; Peaks [Minimum number = 1; Maximum charge = 15]; Aggregation [Scan group aggregation method = Sum].

MS/MS Processing: Un-centroiding [Peak half width = 0.025; Data points per Da = 100; Data points per Da = 100; Always uncentroid = enabled]; Re-gridding [Data points per Da = 100]; Multi-Format Spectrum [Preferred type = Profile]; Peaks [Minimum number = 8; Maximum charge = N/A; Use precursor charge as maximum = enabled]; Precursor Charge [1st choice = Try to re-determine charge from parent scan; 2nd choice = If available, take charge from file; 3rd choice = Use default charge(s); Default charge range = 2 to 15; Ignore singly charged precursors = not enabled]; Precursor Selection [Search within m/z tolerance of = 2.5 Da; Re-determine precursor m/z value when possible = enabled; Maximum number of precursor m/z values = 1]; Aggregation [Scan group aggregation method = Time Domain].

Time Domain: Group assignment [Minimum precursor mass (Mr) = 700; Maximum precursor mass (Mr) = 16 000; Precursor m/z tolerance = 0.01; Maximum intermediate time (secs) = 0; Maximum intermediate scan count = dimmed; Use intermediate scan count when possible = not enabled]; Group Filtering [Minimum number of scans = 1]. Group combination [Sum MS_n scans into MS2 = enabled].

MS Peak Picking: Filtering [Correlation threshold (Rho) = 0.5; Minimum signal to noise (S/N) = 5; Minimum peak m/z = 50; Maximum peak m/z = 100 000]; Peak profile [Minimum peak width (Da) = 0.001; Expected peak width (Da) = 0.025; Maximum peak width (Da) = 0.5; Reject width outliers

= not enabled]; General [Apply baseline correction = not enabled; Fit method = Isotope Distribution; Maximum peak iterations per scan = 500]; Single Peak Window [Pick single peaks in this range (e.g. reporter ions) = not enabled; other parameters dimmed].

MS/MS Peak Picking: General [Same as MS Peak Picking = enabled; other parameters dimmed].

PEAKS Studio 8.5 processing parameters (typical)

Create Project options: [Instrument type = Orbitrap (Orbi-Orbi); Fragment = HCD]. Data

Refinement options: [Merge Scans = not enabled (except for intact #1185 dimer sample); Correct Precursor = enabled; Mass only (recommended) = enabled; Filter Scans = not enabled].

Identification options: [Error Tolerance: Precursor mass = 20 ppm (digested samples)/1.2 Da (intact samples) using monoisotopic mass; Fragment ions = 0.05 Da (digested samples)/1.2 Da (intact samples); Enzyme = None; Selected Fixed PTM = none; Selected Variable PTM = Oxidation (M), Pyro-glu from E, and various “dimer PTMs”; max variable PTM per peptide = 3 to 6; Database = custom made Abeta1-43, APP human isoforms; Uniprot_SwissProt].

TPP/Kojak processing parameters

Gunnar to provide info

Xtract processing parameters (typical)

Resolution@400 = 50 000; S/N Threshold = 2; Fit Factor = 75%; Remainder = 25%; Average Table
= Average; Max Charge = 6. Generate Masses Mode = [M+H]⁺.

Algorithm for combining deconvoluted peak lists from PEAKS Studio and Xtract

While PEAKS Studio was superior to Xtract to deconvolute peaks in the lower *m/z* segment Xtract was slightly better in for larger ions. Apart from that neither program produced a peak list incorporating all peaks produce by the other program, the calculated peak areas were different. The areas were not used for quantification but the actual numbers differed enough making it impossible to show the in the same graph, so a simple harmonization was required. This was done by choosing one common low mass and one common high mass peak from the output of the respective programs, calculating the Xtract-to-PEAKS Studio area ratios, calculating intercept and slope for a line containing the two ratios, and applying this mass-dependent normalization factor to the PEAKS Studio area numbers. The two peak lists were then combined.