Direct Evidence of the Presence of Cross-Linked Aβ Dimers in the Brains of Alzheimer's Disease Patients

Aurelio Vázquez de la Torre,[1][#] Marina Gay,[2][#] Sílvia Vilaprinyó-Pascual,[1] Roberta Mazzucato,[1] Montserrat Serra-Batiste,[1] Marta Vilaseca,[2] Natàlia Carulla[1,3*]

[1]Institute for Research in Biomedicine (IRB Barcelona), The Barcelona Institute of Science and Technology (BIST), Baldiri Reixac 10, 08028 Barcelona, Spain

[2]Mass Spectrometry and Proteomics Core Facility, Institute for Research in Biomedicine (IRB Barcelona), The Barcelona Institute of Science and Technology (BIST), Baldiri Reixac 10, Barcelona 08028, Spain

[3]CBMN (UMR 5248), University of Bordeaux – CNRS – IPB, Institut Européen de Chimie et Biologie, 2 rue Escarpit, 33600, Pessac, France

[#]Both authors have contributed equally to this work

[*]Corresponding author:

Natàlia Carulla, 2 rue Escarpit, 33600, Pessac, France, telephone: +33 (0) 5 4000 3008, e-mail: n.carulla@iecb.u-bordeaux.fr

Abstract

Brain-derived amyloid-β (Aβ) dimers are associated with Alzheimer´s disease (AD). However, their covalent nature remains controversial. This feature is relevant, as a covalent cross-link would make brain-derived dimers (brain dimers) more synaptotoxic than Aβ monomers and would make them suitable candidates for biomarker development. To resolve this controversy, we here present a three-step approach. First, we validated a type of synthetic cross-linked Aβ (CL Aβ) dimers, obtained by means of the photo-induced cross-linking of unmodified proteins (PICUP) reaction, as well-defined mimics of putative brain CL Aβ dimers. Second, we used these PICUP CL Aβ dimers as standards to improve the isolation of brain Aβ dimers and to develop state-of-the-art mass spectrometry (MS) strategies to allow their characterization. Third, we applied these MS methods to the analysis of brain Aβ dimer samples allowing the detection of the CL $[Aβ(6\text{-}16)]_2$ peptide comprising a dityrosine cross-link. This result demonstrates the presence of CL Aβ dimers in the brains of patients with AD and opens up avenues for establishing new therapeutic targets and developing novel biomarkers for this disease.

Introduction

Amyloid-β (Aβ) oligomers are considered the pathogenic molecular form of Aβ in Alzheimer's disease (AD). In this regard, many laboratories worldwide have channeled efforts to either prepare Aβ oligomers using a range of *in vitro* conditions[1-5] or to isolate them from brain tissue.[6,7] As a result of these works, many Aβ oligomer forms have been put forward as the pathogenic form of Aβ in AD. However, establishing which of them is the most pathophysiologically relevant is still a matter of debate.[8] Of the Aβ oligomers described to date, only Aβ dimers have been isolated from the cerebral cortex of subjects with AD. Notably, brain-derived Aβ dimers (brain Aβ dimers) impair synapse structure and function,[7] induce neurofibrillary degeneration,[9] and are associated with this disorder.[10,11] On the basis of these observations, brain Aβ dimers have been proposed as the most pathophysiological relevant material for further pathway analysis and for the preclinical validation of agents designed to neutralize Aβ aggregates.[7]

However, if brain Aβ dimers are to be targeted, they first need to be characterized. In this context, one of the biggest challenges has been to establish whether these dimers are non-covalently or covalently linked. Settling this question is critical because work with synthetic cross-linked Aβ (CL Aβ) dimers has revealed that the cross-link leads to the formation of soluble synaptotoxic assemblies that persist for longer periods than those formed by monomeric Aβ.[12,13] Moreover, demonstration that brain Aβ dimers are cross-linked would facilitate their isolation and manipulation from biological fluids, thus making them suitable candidates for biomarker development.

Two premises support the hypothesis that brain Aβ dimers are cross-linked. First, previous studies have shown that dimers isolated from AD brain tissue are highly

resistant to denaturing conditions.[7,10,14] Second, other studies have reported that cross-linking occurs as a result of oxidative stress, which has also been described as an early event in AD[15]. In this context, immunohistochemical detection of dityrosine (Y-Y) cross-linking has been reported in brain regions enriched in amyloid plaques and in the cerebrospinal fluid (CSF) of AD patients.[16] On the basis of these observations, the formation of a Y-Y cross-link between two Aβ molecules has been proposed. However, in spite of these premises, no direct evidence in support of the covalent nature of brain Aβ dimers has been reported.

The lack of such evidence could be taken to mean that brain Aβ dimers are non-covalent; however, aspects such as their limited sample availability and/or their propensity to aggregate encumber their isolation and subsequent characterization. Here we present a three-step approach to overcome these limitations. First, we established the cross-linked positions in synthetic CL Aβ dimers obtained by means of the photo-induced cross-linking of unmodified proteins (PICUP) reaction.[3,17,18] These dimers are mainly cross-linked through a Y-Y bond, involving the only tyrosine residue present in the Aβ sequence, which is tyrosine 10 (Y10). Since this type of cross-link is the same as that proposed to be formed in the brain under oxidative stress, we considered PICUP CL Aβ dimers as well-defined synthetic mimics of putative brain CL Aβ dimers. Second, we used these PICUP CL Aβ dimers to improve their isolation and to characterize them using state-of-the-art bottom-up mass spectrometry (MS). Third, we applied the optimized isolation and MS characterization strategies to analyze Aβ dimer samples obtained from brain tissue of AD patients. This approach provided unequivocal evidence of the presence of CL Aβ dimers in the brains of patients with this condition.

Experimental Section

GdnHSCN-SEC-WB analysis of synthetic Aβ samples

We worked with low molecular weight (LMW), fibril, and PICUP CL Aβ(1-40) and Aβ(1-42) samples, prepared as detailed in the SI Experimental Section "Preparation of synthetic Aβ samples". Appropriate volumes of these samples, corresponding to 248.9 μg of Aβ(1-40) or Aβ(1-42), were freeze-dried and resuspended in 29.3 μL of 6.8 M guanidine thiocyanate (GdnHSCN) (8.5 mg/mL Aβ). They were then sonicated for 5 min and diluted with 20 μL of H_2O to 4 M GdnHSCN (5 mg/mL Aβ). The samples were passed through a 0.45-μm PVDF centrifugal filter (Millipore) and injected into two columns in series—a Superdex 75 10/300 GL-Superdex 200 10/300 GL (GE Healthcare). The columns were equilibrated in 10 mM ammonium acetate pH 8.5, and the samples were eluted into 1-mL fractions at 4ºC at a flow rate of 0.5 mL/min. The concentration of synthetic Aβ species after Size Exclusion Chromatography (SEC) was determined through a UV detector at 220 nm. A calibration curve had been previously generated from known amounts of Aβ species assessed by reversed-phase high performance liquid chromatography (RP-HPLC).[18] Next, 800 μL of each fraction was taken and stored at -80°C. The remaining 200 μL were aliquoted in 10-μL fractions, which was the minimum amount amenable for aliquot preparation. Afterwards the required volume, such that the dimer fraction corresponded to ~10 ng, was taken for all the SEC fractions. It was then lyophilized, reconstituted in 20 μL of 4X Sample Buffer (4X SB), heated at 95°C for 5 min, and analyzed by WB using monoclonal Aβ antibodies 6E10 (1:5000) and 4G8 (1:5000). To calibrate the tandem Superdex 75-Superdex 200 10/300 GL columns, the following standards were used: β-amilase (200 kDa); bovine serum albumin (BSA) (67 kDa); carbonic anhydrase (29 kDa); and cytochrome C (12.4 kDa).

GdnHSCN-SEC-WB analysis of brain Aβ samples

Brain samples were obtained from the Brain Banks of the Institute of Neuropathology of the IDIBELL-University Hospital Bellvitge and of the Hospital Clínic-Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), both in Barcelona, Spain. The study was carried out in accordance to the Spanish Legislation on this matter (Real Decreto Biobanco 1716/2011) and the approval of the local IDIBELL-University Hospital Bellvitge and IDIBAPS ethics committees. Brain donors and/or relatives gave their written informed consent for the use of brain tissue for research purposes. Brain samples from AD patients and those from neuropathological controls without a history of dementia, psychiatric or neurological diseases were processed between 2-10 h after death of individuals and characterized following CERAD criteria[19] and the Braak&Braak scale.[20,21] Detailed characteristics of patients are provided in Table S2. The GdnHSCN extract was prepared as detailed in the SI Experimental Section "Preparation of human brain samples". 300 μL of 6.8 M GdnHSCN extract, corresponding to the insoluble fraction derived from sequential extractions of 0.6 g of brain tissue, were diluted to 4 M GdnHSCN by adding 210 μL of H_2O. The extract was then clarified by centrifugation at 175,000 g and 4°C in a SW55Ti rotor (Beckman Coulter Inc. Brea, CA, USA) for 30 min. The supernatant was then passed through a 0.45-μm PVDF centrifugal filter (Millipore Corp, Billerica, MA, USA). The resulting solution was injected and eluted using the same SEC columns and conditions used to analyze synthetic Aβ samples. Next, 800 μL of the fractions eluting as dimers were taken and stored at -80°C for bottom-up MS characterization. The remaining 200 μL was aliquoted into 45-μL fractions and then lyophilized, reconstituted in 20 μL of 4X SB, heated at 95°C for 5 min, and used for WB analysis with monoclonal Aβ antibodies 6E10 (1:5000) and 4G8 (1:5000). SEC

fractions containing brain Aβ dimers were quantified by reference to known amounts of PICUP CL Aβ(1-40) dimers (Figure S1). From 0.6 g of brain tissue, we obtained two 1-mL fractions eluting at 24 and 25 mL, each containing the highest concentration of dimers, corresponding to 555 ng and 222 ng, respectively.

Identification of CL peptides and positions in PICUP CL Aβ(1-40) dimers

Sample digestion and LC-MS/MS analysis. 2 μg of PICUP CL Aβ(1-40) dimers prepared and purified by SEC as previously described[18] were lyophilized, resuspended in 50 mM ammonium carbonate at pH 7.8, and digested in the presence of 10% weight of trypsin or GluC at 37°C overnight. Afterwards, digestions were quenched by reaching a final concentration of 1% formic acid (FA), and 1 pmol of the resulting peptide mixtures was loaded onto a nanoscale liquid chromatography system coupled to tandem mass spectrometry (nano-LC-MS/MS). A full description of the nano-LC-MS/MS analysis used to study the digested samples is provided in the SI Experimental Section "nanoLC-MS/MS conditions for the identification of cross-linked peptides and positions in PICUP CL Aβ(1-40) dimers".

Data processing. To identify cross-linked peptides and determine cross-link positions, all data were processed using StavroX v3.6.2,[22] kindly provided by Prof. Dr. Andrea Sinz and Dr. Michael Götze (Martin-Luther University, Halle-Wittenberg, Germany). Searches were run against a FASTA database, which contained the Aβ(1-40) sequence. Search parameters included trypsin and GluC enzyme, allowing for two missed cleavage sites, and for methionine oxidation as a dynamic modification. Given the proposed mechanism for PICUP described in the main text, the following cross-linking positions were considered: Y-Y, tyrosine-serine (Y-S), tyrosine-lysine (Y-K), and tyrosine-histidine (Y-H). Peptide mass tolerance was set at 10 ppm and MS/MS tolerance at 20 ppm. To establish the position of the cross-link, peptide spectrum

matches (PSMs) were filtered with a false discovery rate (FDR) < 1% and subsequently validated manually (Table S1).

Bottom-up MS proteomic identification of cross-linked Aβ peptide [Aβ(6-16)]₂ within dimer fractions

SEC fractions corresponding to either PICUP CL Aβ(1-40) dimers or to brain Aβ dimers were used. For the former, we analyzed 3 technical replicates, while for the latter, we worked with 2 biological replicates, AD1 and AD2 (Table S2). As synthetic samples, a volume corresponding to 500 ng of PICUP CL Aβ(1-40) dimers was used. As brain samples, we worked with 650-800 µL, containing between 144 and 444 ng of brain Aβ dimers (Figure S1). The latter were immunoprecipitated as described in the SI Experimental Section "Immunoprecipitation of brain Aβ dimer fractions". Afterwards, both synthetic and brain samples were resuspended in 40 µL of 50 mM NH_4HCO_3 pH 7.8 and digested with 100 ng of trypsin at 37ºC overnight. Digestions were quenched by reaching a final concentration of 1% FA. Samples were dried in a speed-vac and resuspended with 10 µL of 0.1% TFA. They were then analyzed using the nano-LC-MS/MS system described in the Experimental Section "nanoLC-MS/MS conditions for bottom-up MS proteomic identification of cross-linked Aβ peptide [Aβ(6-16)]₂".

Results

PICUP CL Aβ dimers are mainly cross-linked through Y10-Y10

The PICUP reaction has been widely used to prepare CL Aβ oligomers formed during early stages of Aβ aggregation.[3,18] It involves the photo-excitation of ruthenium(II) tris-bipyridyl complex, Ru(II)bpy₃, in the presence of persulfate to generate Ru(III) and a sulfate radical (Figure S2a). Ru(III) is a potent one-electron oxidant with a strong propensity to generate a radical. Within the Aβ sequence, the

side chain of Y10 is the most reactive site to stabilize a radical. Based on the proposed PICUP mechanism,[3,23] the resultant Y radical could proceed to form cross-linked products by reacting with another Y residue (Figure S2b, right). Alternatively, a nearby reducing side chain, such as those of cysteine (C), serine (S), lysine (K), or histidine (H), could also attack the radical (Figure S2b, left). Given these mechanistic considerations, and since C is not present in the sequence of Aβ, the PICUP reaction could lead to the formation of a cross-link between the following amino acid pairs: Y-Y, Y-S, Y-K, and Y-H. However, in spite of these proposed mechanistic considerations, the actual cross-link positions within PICUP CL Aβ dimers have not been established. To elucidate whether cross-links formed by means of the PICUP reaction are physiologically relevant, such as Y10-Y10,[16,24,25] and to facilitate access to well-defined synthetic mimics of putative brain CL Aβ dimers, we proceeded to characterize the cross-link positions within PICUP CL Aβ dimers.

To this end, we first prepared PICUP CL Aβ(1-40) dimers, as recently described.[18] Next, we subjected them to enzymatic digestion using trypsin and GluC to obtain cross-linked peptides. The latter were subsequently analyzed by nanoLC-MS/MS to identify them and to determine the position of the cross-link. Five distinct cross-linked peptides were identified: three homodimers, [Aβ(4-11)]$_2$, [Aβ(6-16)]$_2$, and [Aβ(1-11)]$_2$, and two heterodimers, Aβ(4-11)-Aβ(1-11) and Aβ(6-16)-Aβ(1-16) (Table S1). Notably, all of them corresponded to the N-terminal part of Aβ and contained Y10 in their sequence. To analyze the LC-MS/MS data, we used StavroX, a software package dedicated to identifying cross-link positions in peptides, and considered the four potential positions proposed for the PICUP mechanism Y-Y, Y-S, Y-K, and Y-H. This analysis revealed that in 267 out of 268 MS/MS spectra, corresponding to the five cross-linked peptides identified, Y10-Y10 was the site with

the highest probability of holding the cross-link (Table S1, Figure 1a,c and Figures S3-S6). Only in two spectra obtained for [Aβ(6-16)]$_2$, one identified by the software and the other after manual validation of all the spectra, Y10-S8 was identified with the highest probability. In these two spectra, the MS/MS fragment y$_7$α^{2+} 470.741, specific for Y10-S8 and not compatible with Y10-Y10 was detected (Table S1 and Fig. 1b,d). Y10-Y10 has been proposed to form within brain Aβ dimers in AD brains as a result of oxidative stress.[16,24,25] Therefore, the finding that in most of the MS/MS spectra corresponding to the five CL Aβ peptides (266 out of 268) derived from PICUP CL Aβ(1-40) dimers, the highest probability for the cross-link position was assigned to Y10-Y10, validates the use of PICUP CL Aβ(1-40) dimers as well-defined mimics of putative brain CL Aβ dimers.

PICUP CL Aβ dimers as standards to improve CL Aβ dimer isolation

Next, we used PICUP CL Aβ dimers as standards to establish biochemical tools to improve their isolation. In this regard, we took advantage of our recently reported strategy to purify PICUP CL Aβ oligomers of defined order, including dimers, trimers, and tetramers.[18] This strategy, referred to as GdnHSCN coupled to SEC (GdnHSCN-SEC), is based on two steps. The first involves the use of 6.8 M GdnHSCN, a strong disaggregating treatment, to break all non-covalent Aβ-Aβ interactions, such that only covalent CL Aβ oligomers formed during the cross-linking reaction are preserved. In the second step, SEC is used to fractionate CL Aβ oligomers on the basis of size. This step is carried out at 4°C and using 10 mM ammonium acetate at pH 8.5 as elution buffer to prevent the aggregation of the samples during fractionation.[7] When PICUP CL Aβ(1-40) and Aβ(1-42) samples were studied using this strategy (Figure S7a and Figure 2a), four peaks were detected in the SEC chromatogram. On the basis of ion mobility coupled to mass spectrometry (IM-MS), these peaks had previously been

assigned to monomers and cross-linked dimers, trimers, and tetramers.[18] Next, to confirm that indeed 6.8 M GdnHSCN was able to break non-covalent Aβ aggregates into their constituent monomers and that the conditions used for SEC analysis prevented subsequent monomer aggregation, we carried out two additional controls. The first one involved submitting LMW Aβ(1-40) and Aβ(1-42) samples to GdnHSCN-SEC analysis (Figure S7c and Figure 2c). LMW corresponds to the lowest aggregation state of Aβ, which comprises monomers in rapid equilibrium with low order non-covalent oligomers. The second control involved subjecting synthetic Aβ(1-40) and Aβ(1-42) fibrils (Figure S8) to GdnHSCN-SEC analysis (Figure S7e and Figure 2e). In both cases, a single peak eluting with the same retention volume as monomeric Aβ was detected. These results indicate that this GdnHSCN-SEC strategy is a reliable way to break Aβ aggregates into their constituent covalent units and to prevent their further aggregation during SEC fractionation.

Next, to make the GdnHSCN-SEC strategy applicable to the analysis of brain samples, expected to contain low amounts of Aβ, we incorporated western blot (WB) to the overall sample analysis. Thus, synthetic samples obtained after GdnHSCN-SEC were subsequently analyzed by WB (Figure S7b,d,f and Figure 2b,d,f). The aggregation state of Aβ(1-40) samples detected by WB was the same as that determined by SEC (Figure S7b,d,f). However, the aggregation state of the Aβ(1-42) samples identified by WB sometimes differed from that determined by SEC (Figure 2b,d,f). For example, Aβ(1-42) monomers obtained after GdnHSCN-SEC ran exclusively as monomers (Figure 2d) or either as monomers and dimers (Figure 2b,f). SDS has been reported to affect the aggregation state of Aβ(1-42).[18,26] In this context, various factors could contribute to the observed anomalous behavior of Aβ(1-42) monomers, including the reconstitution process of the lyophilized Aβ samples in SDS

and/or the ratio of Aβ(1-42) to SDS. On the basis of these results, we decided to use only WB to select fractions for subsequent analysis but not to establish the aggregation state of a given sample. In summary, the results obtained by applying the GdnHSCN-SEC strategy to distinct synthetic Aβ samples, involving different degrees of covalent cross-linking and non-covalent self-assembly, indicate that this strategy is a reliable way to break and fractionate Aβ aggregates into their constituent covalent units. Subsequent analysis by WB allows the selection of fractions for further characterization.

Brain Aβ dimers are isolated from the brains of patients with AD

The solubilization of plaque cores using 6.8 M GdnHSCN has been reported.[27] Therefore, we envisioned that the above GdnHSCN-SEC-WB protocol could be extended to the isolation of putative brain CL Aβ dimers. Previous studies based on the isolation and characterization of such dimers in the human brain relied on extracting soluble, membrane-associated, and insoluble fractions by sequential centrifugation of brain homogenates obtained from humans neuropathologically diagnosed with AD.[7] Of these three extracts, brain Aβ dimers were found to be most abundant in the insoluble fraction.[10,11] Since these dimers impair synapse function in the same manner as those obtained directly from the soluble fraction,[7] we chose to work with the insoluble fraction and thus have access to larger amounts of dimers. Accordingly, to establish the presence of CL Aβ dimers in AD brains, we carried out serial extractions of brain tissue from AD patients and analyzed the insoluble fraction, rich in Aβ plaques, using the GdnHSCN-SEC-WB strategy (Figure 2g,h) previously applied to synthetic samples. WB analysis of the SEC fractions revealed bands corresponding to Aβ monomers, dimers and trimers (Figure 2h). The latter had resisted the strong disaggregating treatment and eluted in the same SEC volume as

their synthetic cross-linked counterparts (compare Figure 2h to 2b), thereby suggesting that CL Aβ oligomers were present in this brain sample. The resistance of brain Aβ dimers to strong denaturants had already been reported.[14] Specifically, the use of denaturants such as 6M GdnHCl, 88% formic acid and 2 M thiourea/6M urea had already been used coupled to SEC fractionation to obtain brain Aβ dimers. Starting from 0.6 g of brain tissue and using the GdnHSCN-SEC strategy, we obtained two fractions, eluting at 24 and 25 mL in SEC, with the highest amounts of brain Aβ dimers, each containing 555 ng and 222 ng, respectively (Figure S1). Following WB analysis, we observed that, compared to brain monomeric Aβ, brain Aβ dimers were more strongly immunodetected by 4G8 (residues 17-24) than by 6E10 (residues 3-8) antibodies (Figure 2h). This observation is also made when comparing synthetic Aβ monomers with PICUP CL Aβ dimers (Figure 2b). The lower immunoreactivity of 6E10 for PICUP CL Aβ dimers relative to synthetic Aβ monomers could be explained by the presence of the Y10-Y10 cross-link within synthetic dimer samples near the 6E10 epitope (Figure 2b). The observation of the same behavior for the brain sample could be taken as an indication that brain Aβ dimers also comprise a cross-link near the 6E10 epitope (Figure 2h). The higher immunoreactivity of 4G8 for brain Aβ dimers relative to brain Aβ monomers has already been described previously[14]. Although in the WBs shown in Figure 2h, it appears that 4G8 does not detect brain monomeric Aβ, 4G8 does recognize it when using a lower antibody dilution at the expense of losing resolution for brain dimer bands (Figure S9).

Next, to establish whether brain Aβ dimers were present during the different stages of AD, we analyzed brain samples from four individuals: three pathologically characterized to have different amounts of Aβ burden and one young non-AD control

(Table S2). We carried out fractionation of brain extracts of the frontal/parietal cortices of the four subjects and analyzed the insoluble pellet, rich in Aβ plaques, using the GdnHSCN-SEC strategy. Afterwards, we used WB to analyze SEC fractions eluting at the same volume as synthetic Aβ monomers and PICUP CL Aβ dimers (Figure 3 and Figure S10). This analysis reproduced previous results in the literature[7] and showed that all the samples, except those corresponding to the young non-AD control, contained monomeric Aβ and Aβ dimers. In the literature, SDS-PAGE bands running as Aβ dimers have been assigned to N-terminal extended (NTE) Aβ forms[28,29]. At this point of the investigation, we addressed this possibility using cyanogen bromide (CNBr) (Figure S11). These experiments allowed us to establish that the species eluting as Aβ dimers in our SEC experiments were not NTE Aβ forms.

The presence of CL Aβ dimers in the brains of patients with AD is confirmed by bottom-up proteomics

Next, to obtain direct evidence of the covalent nature of the isolated brain Aβ dimers, we used high-resolution MS through bottom-up proteomics. To this end, we first enriched SEC fractions eluting as dimers by immunoprecipitation with 4G8 antibody. The antibody was chosen on the basis of previous results showing that 4G8 shows better immunodetection performance for Aβ dimers than 6E10 (Figure S7b Figure 2b and 2h). Afterwards, brain Aβ dimer samples were trypsin-digested and subsequently analyzed by nanoLC-MS/MS. If brain Aβ dimers were cross-linked, this approach should allow us to detect CL peptides encompassing parts of the dimer sequence. One of the most abundant trypsin cross-linked peptide identified within PICUP CL Aβ(1-40) dimers was [Aβ(6-16)]$_2$ (Table S1 and Figure 1). This peptide includes the Y10-Y10 cross-link proposed to occur in an AD brain as a result of oxidative stress.[3,17,18] Moreover, taking into account the diversity of Aβ sequences in

the brain and reports describing a number of N-terminally truncated Aβ forms,[30-32] we envisioned that the detection of [Aβ(6-16)]$_2$ peptide in brain samples was a solid strategy through which to obtain direct evidence of the presence of CL Aβ dimers in the brain.

However, before analyzing immunoprecipitated brain Aβ dimer samples, we optimized the nanoLC-MS/MS strategy by working with PICUP CL Aβ(1-40) dimers. First, we optimized the LC step. Previous analysis of the cross-link position in the synthetic dimers was done using a silica based-C18 chromatographic column, in which the [Aβ(6-16)]$_2$ peptide eluted as a broad peak. Given that we expected brain Aβ dimers to be of low abundance in the brain, we worked to increase the sensitivity of our LC step. To this end, we used an ethylene bridge hybrid (BEH) C18 column, under which the [Aβ(6-16)]$_2$ peptide eluted as a narrow peak with a width-at-half-height of 0.05 min. Afterwards, we set up a targeted proteomics approach based on parallel reaction monitoring (PRM) to increase sensitivity. In this approach, a specific precursor ion is selected, and the full MS/MS spectra is monitored over the whole chromatogram. Specifically, we selected the most abundant + 5 charge state of the [Aβ(6-16)]$_2$ peptide as the precursor ion, m/z 534.8444 and monitored the full MS/MS spectra (Figure 4a,b,e,f-top). Analysis of the 2267 MS/MS spectra obtained for the PICUP CL Aβ(1-40) dimer sample using StavroX revealed Y10-Y10 as the site with the highest probability for the cross-link position (Table S3, Figure 4d,h-top). No fragment ion characteristic for Y10-S8 was detected in any of the spectra recorded. This finding was attributed to its low abundance compared to Y10-Y10 and to the use of different chromatographic conditions.

Next, we analyzed IP-brain Aβ dimer samples corresponding to AD1 and AD2 patients (Table S2) using the optimized nano-LC-MS/MS method. Notably, the same

precursor ion and MS/MS spectrum, as that detected for trypsin digested PICUP CL Aβ40 dimer samples, were detected in both brain Aβ dimer samples (Figure 4a,b,e,f-bottom mirror and Figure S12). To further compare the MS/MS spectra between synthetic and brain dimer samples, we selected four characteristic MS/MS transitions for the [Aβ(6-16)]$_2$ peptide, namely b_2^+ 253.093, y_5^{2+} 324.682, b_{10}^{4+} 631.775, and b_6^{3+} 674.949. The same retention time and relative intensity was detected for the selected MS/MS transitions when comparing those obtained for PICUP CL Aβ(1-40) dimer samples with those obtained for both brain dimer fractions (Figure 4c,g). Analysis of the 119 MS/MS spectra obtained for sample AD2 and of the 11 MS/MS spectra for sample AD1 (Table S3) using StavroX revealed Y10-Y10 as the site with the highest probability of holding the cross-link in the brain Aβ dimer samples (Figure 4d,h-bottom). All together, these results show that the [Aβ(6-16)]$_2$ peptide comprising a cross-link between Y10-Y10 is present in Aβ dimer fractions from the brains of AD patients, thus demonstrating the presence of CL Aβ dimers in the brains of these patients.

Discussion

Throughout this work, we have resolved one of the most controversial questions posed in the literature regarding the nature of brain Aβ dimers.[7,12,13,16] We have unequivocally established that they are cross-linked. This conclusion has been achieved by first using the GdnSCN-SEC-WB protocol to isolate brain Aβ dimers (Figure 2h). Secondly, a bottom-up MS analysis of brain Aβ dimer fractions led to the detection of the [Aβ(6-16)]$_2$ CL peptide comprising a cross-link between Y10-Y10 (Figure 4). This peptide is characteristic of a CL Aβ dimer sequence providing the first direct evidence of the presence of CL Aβ dimers in the brains of patients with AD.

In the brain, covalent dimers have been proposed to form in two ways, namely via the generation of an isopeptide bond between glutamine (Q) and K mediated by transglutaminase[33] or via phenolic coupling of two Y residues mediated by oxidative stress.[16,24,25] Taking into account these two possibilities for *in vivo* cross-linking, various synthetic Aβ dimers have been proposed as mimics of brain ones. Early work by others used crude mixtures containing both CL and non-CL species, also lacking definition of the aggregation state and the site of cross-linking.[33-35] Later on, dimers were formed by the substitution of serine 26 (S26) by cysteine and subsequent disulfide bond formation, [Aβ(M1-40)S26C]$_2$.[36] However, since [Aβ(M1-40)S26C]$_2$ lacks biological relevance, other more relevant synthetic dimers have been produced and studied, including [Aβ(1-40)]$_2$Y-Y,[12] [Aβ(M1-40)]$_2$Y-Y,[37] [Aβ(M1-42)]$_2$Y-Y, and [Aβ(M1-42)]$_2$Q-K.[13] The biological relevance of PICUP CL Aβ oligomers has been put into question due to the non-physiological conditions associated with the PICUP chemistry. To address this issue, an alternative Copper and Hydrogen Peroxide Induced Cross-Linking of Unmodified Proteins (CHIPUP), which utilizes naturally occurring divalent copper ions, hydrogen peroxide and does not require photo activation, was developed.[38] Notably, the oligomer distribution and the properties of the resulting oligomers obtained by CHICUP were found to be similar to those obtained by PICUP. This observation suggested that although the reagents used in the PICUP reaction were not physiological, they promoted the formation of the same intermediates and products as those formed during oxidative stress conditions. Our results provide direct evidence to support this suggestion. Firstly, both PICUP CL Aβ dimers and brain ones are more strongly immunodetected by 4G8 (residues 17-24) than by 6E10 (residues 3-8) (Figure 2b and 2h), an effect most likely due to the presence of the Y10-Y10 cross-link in both samples. Secondly, both dimers contain

the CL $[A\beta(6\text{-}16)]_2$ peptide in their sequences (Figure 4). Thirdly, for both dimers, analysis of the MS/MS spectra obtained from the $[A\beta(6\text{-}16)]_2$ peptide assigned Y10-Y10 as the highest probability for the cross-link to be (Figure 4 and Tables S1 and S3). On the basis of these results, we propose that PICUP CL $A\beta$ dimers are well-defined mimics of brain ones.

The aggregation properties of synthetic covalent dimers such as $[A\beta(1\text{-}40)]_2$Y-Y,[12] $[A\beta(M1\text{-}40)]$Y-Y,[37] $[A\beta(M1\text{-}42)]$Y-Y, and $[A\beta(M1\text{-}42)]$Q-K[13] have been examined. These studies revealed that the starting dimers lack a defined structure but that they all populate bioactive soluble assemblies for longer periods than the $A\beta$ monomer.[36,37,39] Since the soluble assemblies that these synthetic dimers populate inhibit synaptic plasticity,[37] the authors of those studies proposed that the link between $A\beta$ dimers and AD results from the ability of these dimers to further assemble and form synaptotoxic assemblies that persist for longer periods than those formed by $A\beta$ monomers. Given our findings demonstrating the presence of CL $A\beta$ dimers in the brains of AD patients, the ability of synthetic CL $A\beta$ dimers to further assemble and form synaptotoxic assemblies becomes relevant in the context of AD, thus revealing CL $A\beta$ dimers and the assemblies they form as potential new targets in the treatment of AD.

Finally, there is concern that the buffers used during the isolation and manipulation of $A\beta$ oligomers from biological fluids can affect their aggregation state. The finding that CL $A\beta$ dimers are present in the brains of patients with AD is an important step in the field of biomarker development, as the covalent nature of these samples will allow their manipulation under strong denaturing conditions without disruption of their oligomerization state. Of note, the LC-targeted MS/MS method developed throughout this work could be extended to future studies designed to assess the potential of brain CL $A\beta$ dimers as new AD biomarkers. The potential of CL $A\beta$ dimers as biomarkers

of AD is especially relevant since both Aβ and dityrosine have been detected in the CSF of patients with this disease[16] and CSF samples from patients containing soluble Aβ dimers disrupt synaptic plasticity.[40] In conclusion, here we provide unequivocal evidence of the presence of CL Aβ dimers in the brains of AD patients, thus opening up new avenues for the establishment of therapeutic targets and biomarkers for the treatment and diagnosis of this disease.

References

(1)   Lambert, M. P.; Barlow, A. K.; Chromy, B. A.; Edwards, C.; Freed, R.; Liosatos, M.; Morgan, T. E.; Rozovsky, I.; Trommer, B.; Viola, K. L.; Wals, P.; Zhang, C.; Finch, C. E.; Krafft, G. A.; Klein, W. L. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 6448–6453.

(2)   Kayed, R.; Head, E.; Thompson, J. L.; McIntire, T. M.; Milton, S. C.; Cotman, C. W.; Glabe, C. G. *Science* **2003**, *300*, 486–489.

(3)   Bitan, G.; Teplow, D. B. *Acc. Chem. Res.* **2004**, *37*, 357–364.

(4)   Barghorn, S.; Nimmrich, V.; Striebinger, A.; Krantz, C.; Keller, P.; Janson, B.; Bahr, M.; Schmidt, M.; Bitner, R. S.; Harlan, J.; Barlow, E.; Ebert, U.; Hillen, H. *J. Neurochem.* **2005**, *95*, 834–847.

(5)   Serra-Batiste, M.; Ninot-Pedrosa, M.; Bayoumi, M.; Gairí, M.; Maglia, G.; Carulla, N. *Proc. Natl. Acad. Sci. U.S.A.* **2016**, *113*, 10866–10871.

(6)   Lesné, S.; Koh, M. T.; Kotilinek, L.; Kayed, R.; Glabe, C. G.; Yang, A.; Gallagher, M.; Ashe, K. H. *Nature* **2006**, *440*, 352–357.

(7)   Shankar, G. M.; Li, S.; Mehta, T. H.; Garcia-Munoz, A.; Shepardson, N. E.; Smith, I.; Brett, F. M.; Farrell, M. A.; Rowan, M. J.; Lemere, C. A.; Regan, C. M.; Walsh, D. M.; Sabatini, B. L.; Selkoe, D. J. *Nat. Med.* **2008**, *14*, 837–842.

(8)   Benilova, I.; Karran, E.; De Strooper, B. *Nat. Neurosci.* **2012**, *15*, 349–357.

(9)   Jin, M.; Shepardson, N.; Yang, T.; Chen, G.; Walsh, D.; Selkoe, D. J. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 5819–5824.

(10)   Mc Donald, J. M.; Savva, G. M.; Brayne, C.; Welzel, A. T.; Forster, G.; Shankar, G. M.; Selkoe, D. J.; Ince, P. G.; Walsh, D. M.; on behalf of the Medical Research Council Cognitive Function and Ageing Study. *Brain*

2010, *133*, 1328–1341.

(11) McDonald, J. M.; Cairns, N. J.; Taylor-Reinwald, L.; Holtzman, D.; Walsh, D. M. *Brain Res.* **2012**, *1450*, 138–147.

(12) Kok, W. M.; Cottam, J. M.; Ciccotosto, G. D.; Miles, L. A.; Karas, J. A.; Scanlon, D. B.; Roberts, B. R.; Parker, M. W.; Cappai, R.; Barnham, K. J.; Hutton, C. A. *Chem. Sci.* **2013**, *4*, 4449.

(13) O'Malley, T. T.; Witbold, W. M.; Linse, S.; Walsh, D. M. *Biochemistry* **2016**, *55*, 6150–6161.

(14) Mc Donald, J. M.; O'Malley, T. T.; Liu, W.; Mably, A. J.; Brinkmalm, G.; Portelius, E.; Wittbold, W. M.; Frosch, M. P.; Walsh, D. M. *Alzheimer's Dement.* **2015**, *11*, 1286–1305.

(15) Zhu, X.; Su, B.; Wang, X.; Smith, M. A.; Perry, G. *Cell. Mol. Life Sci.* **2007**, *64*, 2202–2210.

(16) Al-Hilaly, Y. K.; Williams, T. L.; Stewart-Parker, M.; Ford, L.; Skaria, E.; Cole, M.; Bucher, W. G.; Morris, K. L.; Sada, A. A.; Thorpe, J. R.; Serpell, L. C. *Acta Neuropathol. Commun.* **2013**, *1*, 1.

(17) Fancy, D. A.; Kodadek, T. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 6020–6024.

(18) Pujol-Pina, R.; Vilaprinyó-Pascual, S.; Mazzucato, R.; Arcella, A.; Vilaseca, M.; Orozco, M.; Carulla, N. *Sci. Rep.* **2015**, *5*, 1–13.

(19) Mirra, S. S.; Heyman, A.; McKeel, D.; Sumi, S. M.; Crain, B. J.; Brownlee, L. M.; Vogel, F. S.; Hughes, J. P.; Belle, von, G.; Berg, L. *Neurology* **1991**, *41*, 479–479.

(20) Braak, H.; Braak, E. *Acta Neuropathol.* **1991**, *82*, 239–259.

(21) Braak, H.; Alafuzoff, I.; Arzberger, T.; Kretzschmar, H.; Del Tredici, K. *Acta Neuropathol.* **2006**, *112*, 389–404.

(22)   Götze, M.; Pettelkau, J.; Schaks, S.; Bosse, K.; Ihling, C. H.; Krauth, F.;
         Fritzsche, R.; Kühn, U.; Sinz, A. *J. Am. Soc. Mass Spectrom.* **2012**, *23*, 76–
         87.

(23)   Fancy, D. A.; Kodadek, T. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 1–6.

(24)   Galeazzi, L.; Ronchi, P.; Franceschi, C.; Giunta, S. *Amyloid* **1999**, *6*, 7–13.

(25)   Atwood, C. S.; Perry, G.; Zeng, H.; Kato, Y.; Jones, W. D.; Ling, K.-Q.;
         Huang, X.; Moir, R. D.; Wang, D.; Sayre, L. M.; Smith, M. A.; Chen, S. G.;
         Bush, A. I. *Biochemistry* **2004**, *43*, 560–568.

(26)   Watt, A. D.; Perez, K. A.; Rembach, A.; Sherrat, N. A.; Hung, L. W.;
         Johanssen, T.; McLean, C. A.; Kok, W. M.; Hutton, C. A.; Fodero-Tavoletti,
         M.; Masters, C. L.; Villemagne, V. L.; Barnham, K. J. *Acta Neuropathol.*
         2013, *125*, 549–564.

(27)   Masters, C. L.; Simms, G.; Weinman, N. A.; Multhaup, G.; McDonald, B. L.;
         Beyreuther, K. *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 4245–4249.

(28)   Portelius, E.; Olsson, M.; Brinkmalm, G.; Rüetschi, U.; Mattsson, N.;
         Andreasson, U.; Gobom, J.; Brinkmalm, A.; Hölttä, M.; Blennow, K.;
         Zetterberg, H. *J. Alzheimers Dis.* **2013**, *33*, 85–93.

(29)   Welzel, A. T.; Maggio, J. E.; Shankar, G. M.; Walker, D. E.; Ostaszewski, B.
         L.; Li, S.; Klyubin, I.; Rowan, M. J.; Seubert, P.; Walsh, D. M.; Selkoe, D. J.
         Biochemistry **2014**, *53*, 3908–3921.

(30)   Watt, A. D.; Crespi, G. A. N.; Down, R. A.; Ascher, D. B.; Gunn, A.; Perez,
         K. A.; McLean, C. A.; Villemagne, V. L.; Parker, M. W.; Barnham, K. J.;
         Miles, L. A. *Acta Neuropathol.* **2014**, *127*, 803–810.

(31)   Kummer, M. P.; Heneka, M. T. *Alzheimer's Res. Ther.* **2014**, *6*, 1–9.

(32)   Wildburger, N. C.; Esparza, T. J.; LeDuc, R. D.; Fellers, R. T.; Thomas, P.

M.; Cairns, N. J.; Kelleher, N. L.; Bateman, R. J.; Brody, D. L. *Sci. Rep.* **2017**, *7*, 9520.

(33)   Hartley, D. M.; Zhao, C.; Speier, A. C.; Woodard, G. A.; Li, S.; Li, Z.; Walz, T. *J. Biol. Chem.* **2008**, *283*, 16790–16800.

(34)   Siegel, S. J.; Bieschke, J.; Powers, E. T.; Kelly, J. W. *Biochemistry* **2007**, *46*, 1503–1510.

(35)   Moore, B. D.; Rangachari, V.; Tay, W. M.; Milkovic, N. M.; Rosenberry, T. L. *Biochemistry* **2009**, *48*, 11796–11806.

(36)   O'Nuallain, B.; Freir, D. B.; Nicoll, A. J.; Risse, E.; Ferguson, N.; Herron, C. E.; Collinge, J.; Walsh, D. M. *J. Neurosci.* **2010**, *30*, 14411–14419.

(37)   O'Malley, T. T.; Oktaviani, N. A.; Zhang, D.; Lomakin, A.; O'Nuallain, B.; Linse, S.; Benedek, G. B.; Rowan, M. J.; Mulder, F. A. A.; Walsh, D. M. *Biochem. J.* **2014**, *461*, 413–426.

(38)   Williams, T. L.; Serpell, L. C.; Urbanc, B. *BBA - Proteins and Proteomics* **2016**, *1864*, 249–259.

(39)   Kok, W. M.; Cottam, J. M.; Ciccotosto, G. D.; Miles, L. A.; Karas, J. A.; Scanlon, D. B.; Roberts, B. R.; Parker, M. W.; Cappai, R.; Barnham, K. J.; Hutton, C. A. *Chem. Sci.* **2013**, *4*, 4449–6.

(40)   Klyubin, I.; Betts, V.; Welzel, A. T.; Blennow, K.; Zetterberg, H.; Wallin, A.; Lemere, C. A.; Cullen, W. K.; Peng, Y.; Wisniewski, T.; Selkoe, D. J.; Anwyl, R.; Walsh, D. M.; Rowan, M. J. *J. Neurosci.* **2008**, *28*, 4231–4237.

(41)   Vizcaíno, J. A.; Csordas, A.; del-Toro, N.; Dianes, J. A.; Griss, J.; Lavidas, I.; Mayer, G.; Perez-Riverol, Y.; Reisinger, F.; Ternent, T.; Xu, Q.-W.; Wang, R.; Hermjakob, H. *Nucleic Acids Res.* **2016**, *44*, D447–D456.

(42)   Mukherjee, S.; Kapp, E. A.; Lothian, A.; Roberts, A. M.; Vasil'ev, Y. V.;

Boughton, B. A.; Barnham, K. J.; Kok, W. M.; Hutton, C. A.; Masters, C. L.; Bush, A. I.; Beckman, J. S.; Dey, S. G.; Roberts, B. R. *Anal. Chem.* **2017**, *89*, 6136–6145.

Supporting Information

Supporting Information Available: Supporting Experimental Section including description of reagents and antibodies, synthetic and human brain samples' preparation, protocol for WB analysis, nano-LC-MS/MS conditions for the identification of CL peptides and positions in PICUP CL Aβ(1-40) dimers, the protocol for immunoprecipitation of brain Aβ dimer fractions, nano-LC-MS/MS conditions for bottom-up MS proteomic identification of cross-linked Aβ peptide [Aβ(1-16)]$_2$, and protocol for cyanogen bromide cleavage. Supporting Figures including quantification of brain Aβ dimers isolated from the GdnSCN extract after SEC-WB analysis, proposed mechanism for the PICUP reaction, identification of the CL positions for all the peptides obtained after either GluC or trypsin digestion of PICUP CL Aβ(1-40) dimer, analysis of various Aβ(1-40) samples using the GdnHSCN-SEC strategy, electron micrographs obtained for the Aβ fibrils used in this study, analysis of the GdnHSCN-SEC fractions of the insoluble fraction of an AD brain with 4G8 antibody at lower dilution, WB analysis of GdnHSCN-SEC fractions eluting as brain Aβ monomers and brain Aβ dimers and corresponding to brains of individuals with different degrees of Aβ burden using 4G8 and 6E10 antibodies, CNBr analysis to indicate that NTE Aβ forms are not detected in the insoluble fraction of brain homogenates analyzed by the GdnHSCN-SEC strategy, expansion panels showing the isotope resolution of the fragment ions of spectra collected from brain samples. Supporting Tables including cross-linked peptides identified and

assignment of cross-link positions in PICUP CL Aβ(1-40) dimers, neuropathological information on the brain samples used in this study, cross-linked positions detected within synthetic and brain Aβ dimer samples.

Acknowledgements

Conflicts of Interest
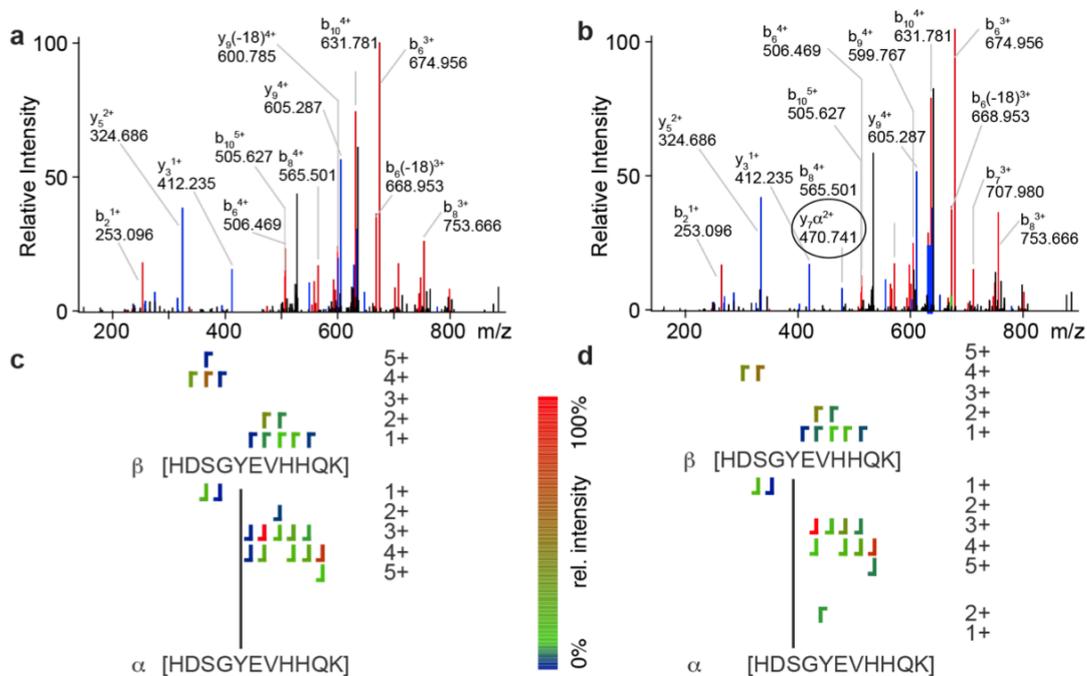
The authors declare no conflicts of interest

Figure 1. Identification of the CL positions within the $[A\beta(6-16)]_2$ peptide obtained after trypsin digestion of PICUP CL $A\beta(1-40)$ dimer. (a, b) Annotated MS/MS spectra, selecting the +5 charge state (m/z 534.8444) of $[A\beta(6-16)]_2$ as precursor ion for CID fragmentation on a Fusion Lumos Orbitrap, for (a) a representative spectrum, out of the 75 detected (a total of 77 measured), consistent with Y10-Y10 and (b) a representative spectrum, out of the 2 detected (a total of 77 measured), consistent with Y10-S8. The MS/MS fragment ion, $y_7\alpha^{2+}$ 470.741, specific for Y10-S8 and not compatible with Y10-Y10 is shown circled. (c, d) Assignment of the fragment ions detected in MS/MS spectra shown in a for c and in b for d analyzed by StavroX, on the peptide sequence. We named the produced fragment ions containing both the cross-link and the two peptide chains using the Schilling nomenclature,[42] which designates the longer chain as α and the shorter as β. However, in cases when due to the symmetry of the CL peptides, the fragment ions could be derived indistinctively from either chain, the α and β label was removed for clarity.
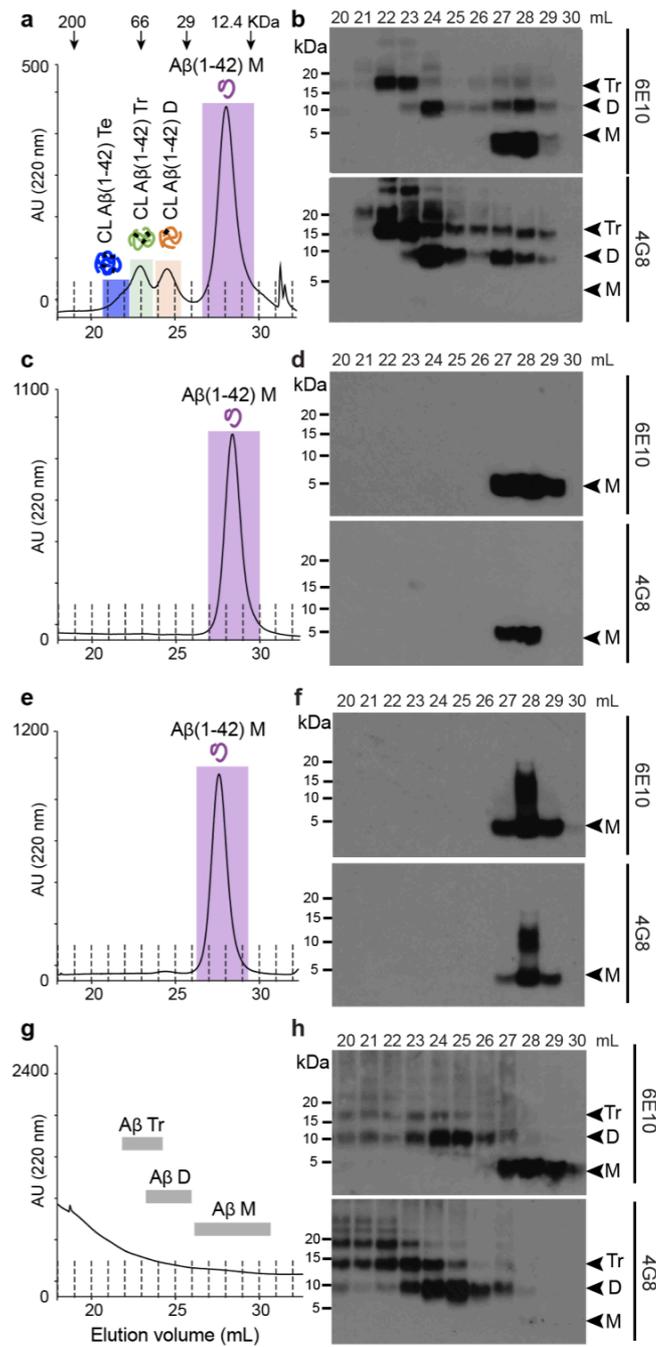
Figure 2. CL Aβ dimers can be isolated by SEC after a harsh disaggregation step. Application of the GdnHSCN-SEC strategy to the analysis of (a) a PICUP CL Aβ(1-42) sample, (c) a LMW Aβ(1-42) sample, (e) an Aβ(1-42) fibril sample, and (g) the insoluble fraction of an AD brain. WB analysis of SEC fractions obtained after GdnHSCN-SEC analysis of (b) a PICUP CL Aβ(1-42) sample, (d) a LMW Aβ(1-42) sample, (f) an Aβ(1-42) fibril sample, and (h) the insoluble fraction of an AD brain.

Samples were blotted with either 6E10 or 4G8 antibodies. M = monomers, D = dimers, Tr = trimers, Te = tetramers.
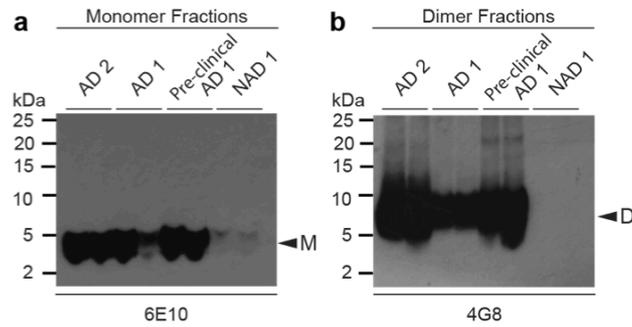
Figure 3. Brain Aβ dimers are detected in the brains of individuals with varying degrees of Aβ burden. The insoluble fraction corresponding to brain homogenates of individuals with distinct degrees of Aβ burden (Table S2) were analyzed using the GndHSCN-SEC strategy. SEC fractions eluting as (a) brain Aβ monomers and (b) brain Aβ dimers were analyzed by WB using 6E10 and 4G8 antibodies, respectively. M = monomer, D = dimer. AD = Alzheimer's disease, Pre-clinical AD = Pre-clinical Alzheimer's disease, NAD = non-Alzheimer's disease.
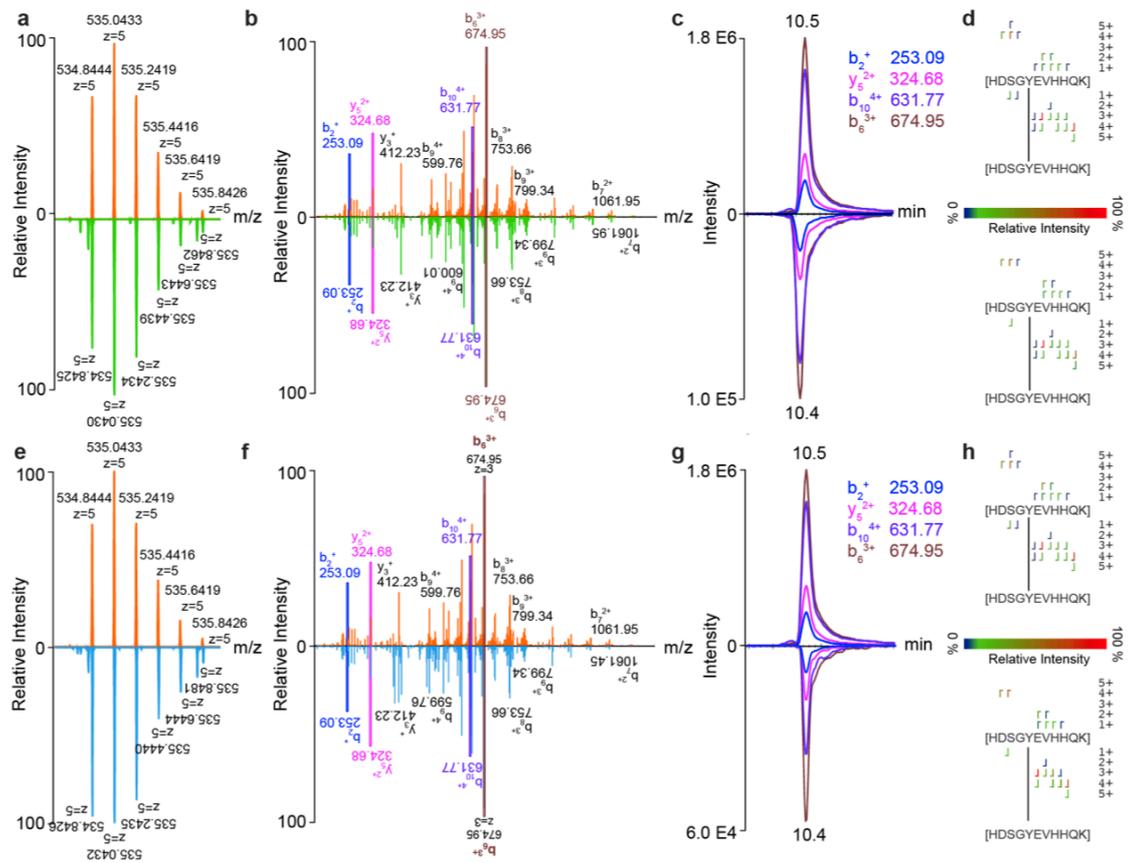
Figure 4. The presence of CL Aβ dimers in the brains of patients with AD is confirmed by bottom-up proteomics. Detection of the trypsin cross-linked peptide [Aβ(6-16)]$_2$ in PICUP CL Aβ(1-40) dimers (a-h, top) and brain Aβ dimer samples obtained from two distinct AD patients, AD2 (a-d, bottom mirror) and AD1 (e-h, bottom mirror). (a, e) Detection of the + 5 charge state of the [Aβ(6-16)]$_2$ peptide as the precursor ion, m/z 534.8444. (b, f) Full MS/MS. (c, g) Selection of four characteristic MS/MS transitions for the [Aβ(6-16)]$_2$ peptide: b_2^+ 253.093, y_5^{2+} 324.682, b_{10}^{4+} 631.775, and b_6^{3+} 674.949. (d, h) Assignment of the fragment ions detected in MS/MS spectra and analyzed by StavroX, on the peptide sequence.

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