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The aqueous phase of Alzheimer's disease brain contains assemblies built from ~4 and ~7 kDa A β species

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Abstract

Much of our knowledge about A β aggregation and toxicity has been acquired using synthetic peptides and mouse models, whereas less attention has been paid to soluble proteins present in human brain. Here we report that aqueous extracts of AD brain can contain at least four different A β assembly forms which include: (i) monomers, (ii) a ~7kDa A β species, and larger species which ranged (iii) from ~30–150 kDa, and (iv) from ~160–700 kDa. The ~30–150 kDa species are by far the most prevalent and appear to be built from ~7 kDa A β species. The component ~7 kDa A β species resist denaturation by chaotropic agents and have a higher A β 42/A β 40 ratio than monomers, and are unreactive with antibodies to Asp1 of A β or APP residues N-terminal of Asp1. These results recommend further analysis of these brain-derived ~7 kDa A β species, the mechanism by which they assemble and the structures which they form.

Keywords

Alzheimer's disease; amyloid β -protein; oligomers; SDS-stable dimer

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Author Contributions

DMW conceived the project, designed experiments, directed research and wrote the paper. JMMcD and TTOM designed and performed experiments, and wrote the paper. WL, AJM and WMW performed experiments. MPF performed histological examination of brain tissue. GB and EP did mass spec analysis. All authors provided critical assessment of the text.

Introduction

Strong genetic evidence indicates that the amyloid β -protein ($A\beta$) plays an initiating role in Alzheimer's disease (AD) (Tanzi, 2012). In recent years much attention has focused on soluble non-fibrillar forms of $A\beta$ loosely referred to as "oligomers" (Klein et al., 2001, Hardy and Selkoe, 2002, Benilova et al., 2012). Literally hundreds of studies have been conducted to investigate the formation, nature and activity of oligomers formed from synthetic $A\beta$, but much less effort has been directed at understanding soluble forms of $A\beta$ present in human brain (Walsh and Teplow, 2012). We and others have shown that aqueous extracts of AD brain contain $A\beta$ species which have disease-relevant bioactivity. In some experiments human brain-derived $A\beta$ preparations were found to be orders of magnitude more potent than synthetic peptides (Shankar et al., 2008, Noguchi et al., 2009, Freir et al., 2011, Jin et al., 2011, Um et al., 2012) and were able to seed amyloidogenesis in amyloid precursor protein (APP) transgenic mice much more efficiently than synthetic assemblies (Langer et al., 2011, Stöhr et al., 2012). Consequently, understanding the assembly forms and composition of AD brain-derived water-soluble assemblies is of great importance.

A number of studies have investigated (usually by ELISA) the relationship between the levels of certain unspecified forms of water-soluble $A\beta$ in brain samples from cases with AD, controls free of amyloid pathology and "controls" with high amyloid pathology. In some reports levels of water-soluble $A\beta$ were selectively elevated in AD (Wang et al., 1999), whereas in others the levels in AD and high amyloid pathology controls were similar (Maarouf et al., 2009, Moore et al., 2012). Using immunoprecipitation (IP) coupled with denaturing SDS-PAGE and Western blotting (WB), we found that the levels of both $A\beta$ monomers and what appeared to be SDS-stable dimers were higher in water-soluble extracts of AD brains than in brains from non-demented individuals or patients with other dementias (Mc Donald et al., 2010). In another widely cited paper, the levels of soluble $A\beta$ in AD and control samples were analyzed by WB and found to correlate significantly with the number of plaques and tangles (McLean et al., 1999). In recent studies denaturing WB'ing revealed that ~4 and ~7 kDa $A\beta$ species, but not slower migrating $A\beta$ immunoreactive bands, increased in AD with increasing Braak stage (Lesné et al., 2013, Watt et al., 2013). Thus, in 6 out of 8 reports, at least some form of water-soluble $A\beta$ was elevated in AD brain. In all such studies, "water-soluble $A\beta$ " was defined operationally as $A\beta$ that was extracted from brain in aqueous detergent-free buffer and remained in solution after high-speed centrifugation. Since water-soluble $A\beta$ was described in this way and because it has been measured using a variety of techniques it is not clear that the species measured in each study were the same. For instance, it is known that certain ELISAs preferentially recognize $A\beta$ monomer and under-detect aggregates of $A\beta$ (Morishima-Kawashima et al., 1995, Enya et al., 1999, Stenh et al., 2005, Yang et al., 2013).

To avoid the confounding issue inherent with assays that cannot discriminate between $A\beta$ monomer and higher assemblies, several groups developed immunoassays that appear not to detect $A\beta$ monomer, and are said to be "oligomer-specific". Using 2 distinct assay formats we found that levels of $A\beta$ oligomers were significantly higher in aqueous brain extracts from patients with AD than in non-demented controls (Xia et al., 2009, Yang et al., 2013)

and a recent study found significantly higher levels of A β oligomers in water-soluble extracts of AD than high amyloid pathology control brains (Esparza et al., 2013). Thus, there is consensus that A β oligomers are present at elevated levels in AD brain, but little is known about the nature of these oligomers.

Here we show that AD brain can contain four or more different sized A β species, the most prevalent of which range from ~30–150 kDa under native conditions. When treated with strong chaotropic agents these 30–150 kDa A β assemblies break down to yield two broad bands that migrate between: (i) 6.1 – 7.9 kDa, and (ii) 3.5 – 4.5 kDa. Quantitative WB'ing using a panel of 9 different anti-A β antibodies demonstrates that both the ~4 and ~7 kDa bands contain multiple different A β primary structures, and that there are significant primary structure differences between the ~4 and ~7 kDa species. Notably, the ~7 kDa material has a much higher A β 42 content and more N-terminally truncated A β alloforms. Importantly, native sizing techniques confirm that the ~4 and ~7 kDa species have distinct physical properties consistent with the ~7 kDa species being a heterogeneous mixture of SDS-stable dimer formed from an array of different monomers. These results lead us to postulate that SDS-stable ~7 kDa A β species are a basic building block of intermediate molecular weight assemblies and that these assemblies play an important role in AD pathogenesis.

Materials and Methods

Reagents and chemicals

Blue dextran was purchased from GE Healthcare (San Francisco, CA) and unbranched dextrans of 5, 12, 25, 80, 150 and 270 kDa were purchased from Pharmacosmos (Holbaek, Denmark). Globular protein size exclusion chromatography (SEC) standards were from Bio-Rad (Hercules, CA). A β 1-40 and A β 1-42 were synthesised, purified and characterised by Dr. J. I. Elliott at Yale University (New Haven, CT). The mass and purity of peptides were determined by electrospray ionization/ion trap mass spectrometry and by reverse phase HPLC, respectively. A β 40 beginning with pyroglutamic acid (A β pE3-40) was a gift from Probiobrug AG (Halle, Germany). A ~7 kDa APP fragment corresponding to A β 1-40 plus the 31 residues of APP immediately N-terminal to Asp1 of A β (–31A β -40) was expressed and purified from *E.coli* (Walsh et al., 2009). Each synthetic peptide (A β 1-40, A β 1-42, A β pE3-40, –31A β -40) was dissolved in 50 mM Tris-HCl, pH 8.5 containing 7 M guanidium-HCl (GuHCl) and 5 mM EDTA at a concentration of 1 mg/ml and incubated at room temperature (RT) overnight to disaggregate pre-existing seeds. Samples were then centrifuged for 30 min at 16,000 g and chromatographed on a Superdex 75 10/300 column eluted at 0.5 ml/min with 50 mM ammonium bicarbonate, pH 8.5. The concentration of the peak fraction for each sample was determined by absorbance at 275 nm (O'Malley et al., 2014). The sample was then diluted to 5 ng/ μ l with 50 mM ammonium bicarbonate, pH 8.5, aliquoted and stored frozen at –80°C. When needed, an aliquot of a given peptide was thawed, used and any remaining sample was discarded. All other chemicals were of the highest purity available and unless indicated otherwise were obtained from Sigma-Aldrich (St. Louis, MO).

Antibodies

The antibodies used and their source are described in Table 1 and in Figure 1.

Preparation and characterization of aqueous extracts of human brain

Human cortical tissue from 36 donors (33 AD, 3 control) was obtained (Table 2) and used in agreement with local Ethics Committee guidelines. All AD cases met current diagnostic criteria based on tangle distribution and plaque burden. Post-mortem intervals were less than 48 hours. Frozen frontal or temporal cortical samples (~1 or ~2 g amounts) were allowed to thaw for ~30 sec at RT and diced into thin slices with a scalpel. Tissue was homogenised in 5 or 10 ml (for 1 or 2 g amounts respectively) of tris-buffered saline (TBS, 20 mM Tris-HCl, pH 7.4 containing 150 mM NaCl, 1 mg/ml pepstatin A, 1 mM pefabloc, 1 mg/ml aprotinin, 5 mM EGTA, sigma phosphatase inhibitor cocktail I and II, and 5 mM EDTA) with 25 strokes of a Dounce homogeniser (Fisher, Ottawa, Canada). Resulting 20% (w/v) homogenates were centrifuged at 150,000 g for 78 min and 4°C in a SW 55 Ti rotor (Beckman Coulter, Fullerton, CA). The upper 90% of the supernatant was removed from each sample, aliquoted and stored at -80°C prior to analysis.

Immunoprecipitations (IP)

In order to minimise detection of cross-reactive proteins that bind non-specifically to Protein A sepharose beads (PrA), samples (10 ml) were incubated with PrA (100 µl each) for one hour at 4°C with gentle shaking on a nutator. PrA was removed by centrifugation (3,500 g for 5 min) and the supernatant divided into 0.5 ml aliquots. A polyclonal antibody (AW7, Table 1) raised against aggregated synthetic Aβ1-42 and that recognises multiple assemblies and epitopes, was used for IP. Briefly, 20, 0.5 ml aliquots were incubated with AW7 plus PrA (15 µl each) on a nutator at 4°C overnight. Aβ-antibody-PrA complexes were collected and washed as previously described (Walsh et al., 2000). PrA pellets were pooled during the last wash step. Aβ was eluted by boiling in 300 µl of 1× sample buffer (50 mM Tris, 2% (w/v) SDS, 12% (v/v) glycerol with 0.01% (w/v) phenol red) and the supernatant collected. 15 µl of supernatant (equivalent to 0.5 ml of starting brain extract) was loaded per gel lane, so that the amount of IP'd material of a given sample was precisely the same on all gels. This approach removes variations in sample detection that could result from using individual IPs for each gel and therefore allows direct comparison between the same sample electrophoresed on separate gels used for WB'ing with different mAbs.

Denaturing sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) and Western blotting

Samples were electrophoresed on hand-poured, 15 well, 16% polyacrylamide tris-tricine gels or pre-cast 26 well, 16.5% polyacrylamide Criterion™ tris-tricine gels (Bio-Rad, Hercules, CA) at 75 V for 15 min and 125 V for an additional 3 hr. Gels were rinsed in transfer buffer (10% methanol, 0.192 M glycine and 25 mM Tris) and electroblotted onto 0.2 µM nitrocellulose membranes at 400 mA and 4°C for 2 hr. Synthetic Aβ standards of equal amounts were run at either ends of each gel to allow comparison of brain samples run on different gels and to ensure even transfer onto the nitrocellulose membrane. To improve sensitivity of detection, membranes were boiled in a microwave oven for 1.5 min at 800 W

in 50 ml phosphate buffered saline (PBS) and allowed to stand for 3.5 min, turned, microwaved again and allowed to stand for an additional 3.5 min (Ida et al., 1996). To further ensure even transfer of protein, the nitrocellulose was stained with Ponceau S (Klein et al., 1995) and assessed by eye. Membranes were blocked with 50% Odyssey blocking buffer in PBS for 1 hr at RT to minimize non-specific binding of primary and secondary antibodies to the nitrocellulose. Blots were then incubated overnight at 4°C with an appropriate primary antibody (Figure 1 and Table 1) in PBS containing 0.05% (v/v) Tween 20 (PBST) and 1% BSA. Membranes were washed six times for 10 min with PBST and then incubated with goat anti-mouse infrared 800 antibody (Rockland, Gilbertville, PA) diluted 1:15,000 in PBS containing 0.02% (w/v) SDS for 1 hr at RT. Membranes were washed six times for 10 min with PBST and then a further two times with PBS, and immunoreactive bands were visualised using a Li-COR Odyssey infrared imaging system (Li-COR, Lincoln, NE).

Since glycosylation of APP at or near the 1G6, Z31 and Pre- β epitopes is possible (Halim et al., 2011) we were careful to test reactivity of APP antibodies on brain extracts treated +/- removal of O-linked sugar groups. For this AD TBS was IP'd with AW7, antigen was released with ammonium hydroxide, and the eluate lyophilized. Thereafter, the lyophilized sample was reconstituted in 45 μ l of deglycosylation mix reaction buffer (Promega, Madison, WI) and incubated at 37°C for 18 hours in the presence or absence of deglycosylation enzyme mix (5 μ l enzyme or 5 μ l reaction buffer added). The deglycosylation mix is comprised of 5 enzymes: PNGase F, O-Glycosidase, Neuraminidase, β 1-4 Galactosidase, β -N-Acetylglucosaminidase which allows for removal of both N and O-linked sugar groups. Thereafter 2 $\times$ sample buffer was added, the sample boiled and analyzed by SDS-PAGE and WB.

Size exclusion chromatography (SEC)

For most experiments, 1 ml of TBS extract or immunoprecipitated TBS extract was chromatographed on a Superdex 75, 10/300 GL column eluted at 0.5 ml/min in 50 mM ammonium acetate, pH 8.5. One ml or 0.5 ml fractions were collected, lyophilized and used for direct immunoblot. For some experiments, 2 ml of TBS extract was injected on to a Superdex 75 10/300 GL column linked to a Superdex 200 10/300 GL column and eluted at 0.8 ml/min. To concentrate and denature A β from TBS extracts, samples were IP'd with AW7 and the concentrate treated with a strong denaturant. Briefly, A β -antibody complex was disrupted by vortexing in 150 mM ammonium hydroxide and the supernatant collected and lyophilized. This material was then re-suspended in a strong chaotrope, typically 50 mM Tris-HCl, pH 10.4 containing 6 M guanidium hydrochloride (GuHCl) and 5 mM EDTA. To assist dissociation of the high and intermediate molecular weight assemblies, samples were boiled for 10 min and incubated at RT for ~18 hr, centrifuged for 30 min at 16,000 g to remove insoluble debris and then chromatographed on a Superdex 75 10/300 GL column as described above.

To test if the SEC elution profile of A β species obtained using 50 mM ammonium acetate, pH 8.5 was comparable to that when a physiological relevant buffer was used, we chromatographed AD brain extracts on the same Superdex 75 column first in 50 mM

ammonium acetate, pH 8.5 and then in artificial cerebrospinal fluid (aCSF), i.e. 1.25 mM sodium dihydrogen phosphate, 26 mM sodium bicarbonate containing 124 NaCl and 2.8 KCl, pH 7.5.

A β 1-x and A β x-42 Enzyme-linked immunosorbant assays (ELISA)

Assays utilised the Meso Scale Discovery (MSD) platform and reagents from Meso Scale (Rockville, MD). MULTI-ARRAY[®] 96 well small-spot black microplates were coated with 3 μ g/ml of monoclonal antibody 266 in tris buffered saline (TBS) and incubated at RT for 18 hr. Monoclonal antibody 266 recognises the mid-region of A β (Fig.1), thus enabling detection of both N- and C-terminally heterogeneous A β species. Binding sites that were unoccupied were blocked in 150 μ l of 5% Blocker A (MSD) in TBS containing 0.05% tween 20 (TBST) and agitated at 400 rpm for 1 hr and 22°C. Plates were washed 3 times with TBST and samples and standards were applied in duplicate, and agitated at 400 rpm for 2 hr and RT. After the capture phase, plates were washed 3 times with TBST and incubated with biotinylated antibody. To allow the detection of A β 1-x or A β x-42, we used biotinylated 3D6 (1 μ g/ml) or 21F12 (1 μ g/ml), respectively. Simultaneously, 1 μ g/ml of the reporter reagent (SULFO-TAG Labelled Streptavidin) was added in ELISA diluent and incubated at RT with gentle agitation for 2 hr. Finally, plates were washed 3 times with PBST and 2 $\times$ MSD read buffer (150 μ l per well) was applied to allow for electrochemiluminescence detection. A SECTOR imager was used to measure the intensity of emitted light, thus allowing quantitative measurement of analytes present in the samples.

Oligomer-specific ELISA (oELISA)

We recently developed an MSD-based assay that reliably detects A β oligomers of a variety of different sizes (Yang et al., 2015). This assay was performed essentially as described above, but employed the novel aggregate-preferring mAb, 1C22, for capture and the Asp1-specific mAb, 3D6, for detection. This oELISA is >37,000-fold more sensitive for A β oligomers than A β monomer (Yang et al., 2015).

Preparation of dityrosine cross-linked A β (DiY) dimer

A β 1-40 or A β 1-42 monomer was oxidatively cross-linked using horseradish peroxidase (HRP) (Wiltfang et al., 1991, Moir et al., 2005, O'Malley et al., 2014). Briefly, A β monomer was isolated using a Superdex 75 10/300 column eluted in 50 mM ammonium bicarbonate, pH 8.5, diluted to 40 μ M and incubated overnight at 37°C in the presence of 2.2 μ M HRP and 250 μ M H₂O₂ (O'Malley et al., 2014). The cross-linking reaction was quenched by addition of 0.02% (w/v) NaN₃ and dimer was isolated from unreacted monomer and aggregates using a Superdex 75 16/600 column eluted in 50 mM ammonium bicarbonate, pH 8.5 (O'Malley et al., 2014).

Asymmetric flow field-flow fractionation (AF4)

Asymmetric field flow fractionation is a chromatography technique in which the separation of the sample is performed in a channel of several hundred μ m height with a semipermeable membrane at the bottom. Because of the laminar flow of the carrier fluid, a parabolic flow profile is created. When a perpendicular (cross) flow is applied to the laminar stream, the

analytes accumulate close to the semipermeable membrane. This concentration build-up causes particle diffusion away from the wall. Smaller particles have higher diffusion and thus have a higher mean elevation above the accumulation wall and elute faster than larger particles. The elution profile is thus the inverse order of SEC. AD-TBS samples were IP'd using AW7, and denatured in GuHCl (as above) and then injected onto an Eclipse DualTec® AF4 (Wyatt Technology, Santa Barbara, CA) and eluted with 50 mM ammonium acetate pH 8.5. The channel was 24.6 cm long and fitted with a 350 µm spacer and a 5 kDa MWCO polyethersulfone membrane. Samples were focused for 6 min during injection at 3 ml/min. The cross-flow was held for 10 min at 3 ml/min before linearly decreasing the flow to zero over 10 min. One ml fractions were collected, lyophilized and used for direct immunoblot.

Mass Spectrometry

MALDI TOF/TOF measurements were performed using a Bruker Daltonics UltraFlex Xtreme instrument (Bruker Daltonics, Bremen, Germany) operated in reflector or linear mode as described elsewhere (Pannee et al., 2014). Nanoflow LC-ESI-LQIT-FTICR measurements were conducted with an Ettan MDLC (GE Healthcare, Uppsala, Sweden), operated with a Zorbax 300 SB-C8 column (Agilent Technologies, Palo Alto, CA, USA), coupled to an LTQ FT Ultra (Thermo Fischer Scientific, Bremen, Germany) hybrid linear quadrupole ion trap–Fourier transform ion cyclotron resonance mass spectrometer as described previously (Brinkmalm et al., 2012). All spectra were acquired in FTICR mode.

Results

We prepared TBS extracts from a total of thirty six donor samples, using cortical tissue from thirty three AD and three control cases (Table 2). Initial analysis employed the pan anti-Aβ antibody, AW7, for IP and a combination of anti-Aβ40 and anti-Aβ42 monoclonal antibodies (mAbs) for WB. To assess the efficiency of the AW7 IP we used 2 different ELISAs; one specific for Aβ beginning at Asp1 (1-x) and another that detects Aβ termination at Ala42 (x-42), to measure Aβ in brain extracts before and after IP. In the eight AD samples, AW7 captured ~90% of all ELISA detectable Aβ (86.8% ± 26.9% of Asp1 Aβ, and 95.6% ± 2.7% of Aβ42). As in our previous work (Shankar et al., 2008, Mc Donald et al., 2010, Borlikova et al., 2013) IP/WB analysis of AD samples revealed two broad bands. By comparison to SeeBlue Plus prestained markers (MWs of 4 kDa, 7 kDa, 16 kDa, 34 kDa, 45 kDa, and 55 kDa), we estimated the apparent molecular weight of each band. Based on analysis of 8 different WBs, each of which contained at least one lane of standards and two lanes of IP'd AD brain TBS extract, we obtained average molecular weights of: (i) 3.5 – 4.5 kDa, and (ii) 6.1 – 7.9 kDa (Fig. 2). We refer to the ~4 kDa band as monomer and the ~7 kDa band as putative dimer or ~7 kDa Aβ. However, we are mindful that SDS-PAGE is not a reliable means to assess native assembly size (Bitan et al., 2005, Hepler et al., 2006) and that using mixtures of mAbs for WB provides no information about the relative abundance of primary structures present in these two bands. Therefore we applied a range of other approaches to investigate the molecular composition and native assembly size of these putative monomers and ~7 kDa Aβ species.

IP/WB demonstrates that AD TBS ~7 kDa A β contain a higher A β 42/A β 40 ratio than A β monomer

To gain insight into the primary structures and relative abundance of A β species present in AD TBS we used a series of sequence-specific antibodies in a quantitative WB approach. Large batches of samples were IP'd with AW7, and equal volumes of IP eluate electrophoresed on SDS-PAGE alongside synthetic A β of known concentration, and WB'd with one of 13 different mAbs (Fig. 3 – 4, Supplementary Fig. 1 – 4). In total, TBS extracts from eight different AD brains and three control brains were analyzed. In order to facilitate comparisons between samples within gels and between samples on different gels we were careful to: (i) include synthetic standards at each end of each gel, and (ii) to ponceau S stain blots to ensure even transfer of protein across blots. No A β was detected in two out of three control brains and in the one extract in which A β was detected (C1) the amount of A β was less than that detected in any of the extracts from AD brains (Supplementary Fig. 2). When probed with mAbs to the unmodified A β sequence one or two specific immunoreactive bands were detected in all of the AD samples (Fig. 3 and Supplementary Fig. 1–3). The most commonly encountered species migrated on SDS-PAGE as a broad band centered at ~4 kDa (Fig. 2) and contained at least 3 electrophoretically distinct species (Supplementary Fig. 1C and D). A broad multi-component putative dimer band centered ~7 kDa was also detected in all AD samples probed with 4G8, 266 and 21F12 (Fig. 3, Supplementary Fig. 2 and 3), and a faint diffuse smear between ~9–30 kDa was seen with mid-region and C-terminal mAbs.

The amount of monomer varied from case to case and depended on the mAb used for detection. The ~4 kDa band frequently resolved to yield three distinct bands (Supplementary Fig. 1C and D, Table 3), and within a sample, N-terminal, mid-region and 40/42-specific mAbs detected varying levels of monomer (Fig. 3 and Supplementary Fig. 1–3). 3D6 detected a single band that co-migrated with A β 1–40 and A β 1–42 standards (Fig. 3A, Supplementary Figs. 1D and 2A, and Table 3). 6E10 and 4G8 detected the same band as 3D6 plus a lower migrating band (Fig. 3B and C, Supplementary Figs. 1D, 2B and 2C, and Table 3), and C-terminal specific mAbs detected the same bands as 6E10 and 4G8 plus a third faster migrating band (Fig. 3D and E, Supplementary Fig. 1, Supplementary Fig. 2D and E, Table 3). These results indicate that the upper monomer band contains A β species that begin at Asp1, the middle band is composed of N-terminally truncated species, and the bottom band is likely p3 (17–40/2). The amount of A β detected by the Asp1-specific mAb, 3D6, varied widely between samples with Asp1 monomer present in 7/8 AD brains (Fig. 3A, Supplementary Fig. 2A and Table 3). 2G3-reactive A β 40 monomer was present in 7/8 samples, but tended to be at lower levels than monomer detected by 6E10, 4G8 or 21F12. When comparing the amount of A β detected in each band by different mAbs it is clear that the monomer and dimer bands have distinct compositions (Table 3). Specifically, A β 40 was detected in monomer from most (7/8) AD brains and was a dominant C-terminal species in 5 of these (Fig. 3, Supplementary Fig. 1 and 2), and although A β 40 was present in dimer bands in 7 of 8 cases it was never a major dimer species (Fig. 3, Supplementary Fig. 1 and 2, and Table 3). In contrast, A β 42 was present in all monomer and dimer bands, with the amount of A β 42 in the dimer exceeding that in the monomer in 7/8 cases (Fig. 3E, and Supplementary Fig. 1B and Supplementary Fig. 2E, and Table 3). Thus in all 8 cases the amount of A β 42 relative to A β 40 was always higher in ~7 kDa A β species than monomers.

Reactivity with the mid-region mAb, 4G8, and lack of detection with (i) an mAb, 46-4, that does not detect A β ; and (ii) AD brain extract IP'd with pre-immune serum, confirm that both the ~4 kDa and ~7 kDa are authentic A β species (Fig. 3C, Supplementary Fig. 2C, Supplementary Fig. 3 and Table 3). It is also noteworthy that a significant portion of A β monomer in all 8 samples was reactive with the N-terminally directed mAb, 6E10 (Fig. 3B and Supplementary Fig. 2B, and Table 3), and that at least some of the monomer in 7 of 8 cases was also detected with the Asp1-specific mAb, 3D6 (Fig. 3A and Supplementary Fig. 2A, and Table 3). As was the case with their C-termini, the composition of A β monomers differed from those of ~7 kDa A β species with regard to N-termini. For instance, a significant portion of monomer in all cases was faintly detected with 6E10, whereas just 3 of the same samples had dimer that was detected by 6E10, and in no case was the amount of 6E10-reactive-dimer higher than the amount of 6E10-reactive-monomer (Fig. 3B and Supplementary Fig. 2B, and Table 3). It is interesting to note that 6E10 does not react with A β phosphorylated at Ser8 (Kumar et al., 2011) and is weakly reactive to A β cross-linked at Tyr10 (T. O'Malley & D. Walsh, unpublished), raising the possibility that at least some of the ~7 kDa dimer could be post-translationally modified.

In a small number of cases certain monomer and dimer bands were detected by all 5 mAbs and although the amount of each epitope varied considerably this may indicate that at least some of the monomers and ~7 kDa A β species contained full length A β 1-40 and/or A β 1-42 (Fig. 3 and Supplementary Figs. 1 and 2, and Table 3). However, only a fraction of all monomers and ~7 kDa A β species contained N-termini detected by 3D6, thus indicating that most AD TBS A β : (i) extends N-terminal from Asp1 (Kaneko et al., 2014, Welzel et al., 2014), (ii) begins after Asp1, or (iii) that the N-terminus is otherwise modified. To address some of these possibilities we used antibodies specific for the presence of pyroGlu3 (pE3) and for APP sequences N-terminal of Asp1 (Fig. 4). We employed 3 antibodies each of which recognize different epitopes within the 30 residues stretch of the APP sequence immediately N-terminal to the A β domain. Since glycosylation of APP at or near these sites could influence antibody recognition we tested reactivity plus and minus treatment to remove N- and O-linked sugars under conditions that readily deglycosylated full length APP (not shown). No N-terminally extended A β (NTE) species were evident in any of the AD TBS extracts examined, whereas the recombinantly produced NTE (-31A β 40) was readily detected by the anti-APP antibodies 1G6 (Fig. 4A and B), Z31 and pre- β (Supplementary Fig. 4A and B). Synthetic pE3 was readily and specifically detected, but pE3 was detected in only one of four brain extracts, and even in that case only a very small amount of pE3 A β was detected (Fig. 4A and C). Importantly, re-probing of blots with 2G3 and 21F12 demonstrated that there was abundant monomer and dimer in samples which did not react with antibodies to NTE or pE3 (Fig. 4 and Supplementary Fig. 4).

Taken together, our quantitative immunoprofiling study indicates that AD TBS contains a heterogeneous mixture of A β sequences and that the relative abundance of these species varies between individual AD cases. Moreover, it is clear that within cases the composition of primary structures of A β differ significantly for A β monomers versus ~7 kDa A β species. In particular, two disease linked changes (Nilsberth et al., 2001, Tomiyama et al., 2008) are

increased in ~7 kDa A β species relative to monomers. The ~7 kDa A β species contains higher A β 42/A β 40 ratios, and more N-terminally modified A β .

Non-denaturing size exclusion chromatography (SEC) of AD TBS reveals the presence of A β in at least 4 different molecular weight fractions, the most prevalent of which ranged from ~30–150 kDa

SDS-PAGE is highly denaturing and can provide little information about native assemblies, and IP could potentially induce formation of the ~7 kDa species, and/or higher aggregates, thus we used non-denaturing SEC to estimate the size of A β assemblies. Aqueous extracts of AD brain were chromatographed in a volatile buffer, fractions collected and concentrated to dryness. Thereafter fractions were electrophoresed on SDS-PAGE and used for WB. Thirty samples were analyzed using SEC (for an example see Fig. 5A), and A β was detected in at least one fraction from each sample (Table 4). In all but two cases A β immunoreactive species were detected in fxs 1, 2 or 3. As fxs 1–3 occur just after the void volume and before elution of ovalbumin (44 kDa) we refer to A β that eluted in these fractions as intermediate molecular weight (Int mwt) A β (Fig. 5A). Roughly one third of AD TBS extracts also contained A β which eluted in fractions with predicted sizes consistent for putative dimers (7/30) and monomers (8/28), and we refer to these as ~7 kDa A β and “true” monomers (Table 4). A portion (9/30) of extracts contained A β that eluted in the void volume, and we refer to this material as high molecular weight (High mwt) A β (Table 4).

Notably, the fractions that contained true monomer migrated on SDS-PAGE exclusively at ~4 kDa, and the fractions that contained putative dimer migrated on SDS-PAGE at ~7 kDa. SEC-isolated High mwt or Int mwt species appeared as low molecular weight species on SDS-PAGE (Fig. 5A and C, Table 4) and no other specific bands were detected (Supplementary Fig. 5). Taken together these results demonstrate that aqueous extracts of AD brain contain at least 4 different sized fractions: true monomer and ~7 kDa A β species which migrate appropriately on SDS-PAGE; Int mwt and High mwt A β which are SDS labile and break down to A β monomers and ~7 kDa A β species.

In a pilot experiment extract from AD30 was chromatographed on a Superdex 75 column eluted in aCSF or in ammonium acetate, pH 8.5. Since aCSF is not volatile and fractions eluted in this buffer are not amenable to lyophilization and subsequent WB we detected A β using ELISAs. We employed the A β x-42 ELISA, which preferentially recognizes A β monomers, and an ELISA specific for oligomers that contain at least one 3D6-reactive component (Yang et al., 2015). The oELISA signal was strongest in fxs 1–5 (Fig. 6A and B), whereas the A β x-42 ELISA detected a weak signal in fxs 1–4 and a strong signal in fxs 14–18 (Fig. 6C and D). The pattern was the same in both elution buffers. Two prominent A β species were detected: the major component eluted as Int mwt A β and the minor component as A β monomer. Moreover, when ammonium acetate fractions were lyophilized and used for WB the bulk of the A β was found in fxs 1–3 as bands that migrated on SDS-PAGE at ~4 and ~7 kDa (Fig. 7A).

In an effort to improve resolution of larger molecular weight assemblies we used a Superdex 75 column linked in series to a Superdex 200 column (Supplementary Fig. 6), and analyzed three AD extracts, each which we knew to contain at least 3 different sized A β species. We

used linear dextran standards to predict the molecular weight of small A β species, since it is known that A β monomer has an extended conformation, the size of which is best estimated by reference to structurally extended linear dextrans (Paivio et al., 2004). This approach confirmed that true monomers and putative ~7 kDa dimers eluted as expected with peak fractions having masses of ~4 – 5 kDa and ~8 – 12 kDa (estimated by reference to linear dextrans), respectively (Fig. 5C). The High mwt species which eluted in the void of a single superdex 75 column was now detected in the included volume of the combined columns and eluted in a range consistent with globular proteins of ~160–700 kDa and had a molecular weight of 150–300 kDa in comparison to linear dextrans (Fig. 5C). In accord with our initial SEC analysis (Table 4, Fig. 5A), the Int mwt species in the three samples analyzed on the tandem system spanned a mass range of ~25–150 kDa based on linear dextrans and 30–150 kDa based on globular proteins (Fig. 5C, Table 4).

SDS-stable ~7 kDa A β species are an important component of intermediate molecular weight A β assemblies

Our SEC studies indicate that Int mwt A β is by far the most commonly encountered A β species in aqueous extracts of AD brain (Table 4). On denaturing SDS-PAGE this material breaks down to yield species that migrate with molecular weights expected for A β monomers and ~7 kDa A β species (Fig. 5 and Table 4). However, it is not clear if the SDS-PAGE ~7 kDa band represents a true A β dimer, or an aberrantly migrating A β monomer. Indeed, several studies have found that a fraction of synthetic A β migrates anomalously on SDS-PAGE (Bitan et al., 2005, Hepler et al., 2006, Watt et al., 2013). Bearing in mind that our quantitative WB experiments revealed that ~7 kDa A β species contained more A β 42 than did monomers (Fig. 3 and Supplementary Fig. 1 and 2) it is conceivable that the ~7 kDa band was not actually a dimer, but an aberrantly migrating N-terminally truncated A β 42 monomer. Since we know that natively isolated High mwt/ Int mwt A β breaks down on SDS-PAGE to yield ~7 kDa species, we reasoned that if we first denatured High mwt/Int mwt A β we could then use native SEC to estimate the size of the A β species produced by breaking up native High mwt/Int mwt A β assemblies. Thus we undertook a series of experiments that combined the use of strong chaotropes and SEC. GuHCl, formic acid and urea were used to dissociate High mwt/Int mwt A β and then non-denaturing SEC was employed to size the breakdown products (Fig. 7). When the TBS extract of AD brain 30 was chromatographed on a Superdex 75 column only High/Int mwt A β was detected (Fig. 7A). These species migrated on SDS-PAGE at ~4 kDa and ~7 kDa, (fxs 1–2). Whenever the same extract was IP'd with AW7, treated with strong chaotropes and then chromatographed, little or no High mwt A β was detected and there was a substantial increase in the amount of ~4 and 7 kDa A β species (Compare Fig. 7A with Fig. 7B–D). Importantly, similar results were obtained with 5 different brains (not shown) and when SEC fractions of AD brain 30 were analyzed by ELISA (Supplementary Fig. 7). In AD brain 30 extract not treated with denaturant A β oligomers were readily detected in fxs 1–4, whereas prior incubation of the brain extract with GuHCl abolished the High mwt signal and produced a greatly enhanced monomer signal (fxs 14–18), and a new signal appeared in spanning fxs 2–13 (Supplementary Figure 7C and G). These ELISA results nicely complement our WB experiments (Fig. 7A–D) from which it is apparent that the major species in AD brain are relatively High mwt oligomers, but that upon treatment with denaturants these break down

to yield: (1) low mwt species which behave on SDS-PAGE and SEC as A β monomer and SDS-stable dimers; and (2) Int mwt species which migrate on SDS-PAGE as putative dimers.

Moreover, ~7 kDa A β species and true monomer from both native and chaotrope-treated AD brain extracts eluted in the same fractions as synthetic dimer (fxs 3–5), and monomer (fxs 6 and 7) (Fig. 8A and B). Thus, given that the major chaotrope-induced breakdown products of Int mwt A β co-eluted from SEC with synthetic cross-linked dimer (Fig. 8) it is reasonable to assume that these brain-derived species are authentic A β dimers.

In an orthogonal approach, we then used quantitative WB'ing to compare the primary structure of monomer and dimer isolated by SEC of denatured AD TBS. Five ml of AD TBS (AD33) was immunoprecipitated using AW7, eluted in 150 mM ammonium hydroxide (AmOH), lyophilized and dissolved in 6M GuHCl. The resulting sample was chromatographed, 0.5 ml fractions collected (to maximize species resolution on a single Superdex 75 column) and a portion of each fraction (100 μ l) lyophilized and analyzed by WB. 2G3/21F12-reactive material that migrated on SDS-PAGE at ~7 kDa was detected in fxs 2–13, and ~4 kDa monomer was detected in fxs 14–17 (Fig. 9A). The presence of SDS-stable dimers across such a wide molecular weight range indicates incomplete disassembly of the High/ Int mwt species present in this particular brain extract (compare SEC of native AD33 extract in Fig. 5A vs. denatured AD33 extract, Fig. 9A). Nonetheless this profile allowed us to compare the primary structure of A β species present in different sized assemblies built from SDS-stable dimers. To this end we pooled fractions from 5 distinct molecular weight ranges (intermediate mwt dimer 1, fxs 2–4; intermediate mwt dimer 2, fxs 5–7; intermediate mwt dimer 3, fxs 8–10; dimer, fxs 11–13; monomer, fxs 15–17) (Fig. 9A). The N-terminal anti-A β mAbs 3D6 and 6E10 readily detected A β monomer, but none of the SDS-stable dimers (Fig. 9B). In contrast, a combination of 2G3 and 21F12 readily detected both A β monomer and the 4 different pools of dimer (Fig. 9B). The results obtained with SEC-isolated monomers and dimers from denatured AD brain extract (Fig. 9B) are entirely consistent with our IP/WB results (Fig. 3 and Supplementary Fig. 2). These results also jibe with the finding that High mwt and Int mwt aggregates built of A β monomers and ~7 kDa A β are detected by the 3D6-based oELISA, whereas oligomers composed solely of ~7 kDa A β (which we know to be N-terminally truncated) are not detected by the oELISA (Supplementary Fig. 7). Specifically, when a TBS extract from AD brain 33 was IP'd with AW7; monomer, but not dimer was detected when 3D6 and 6E10 were used for western blotting, whereas both monomer and dimer were detected with 4G8, 2G3 and 21F12 (Supplementary Fig. 2).

Since interpretation of molecular weight by SEC can be confounded by non-ideal interactions with it's stationary phase (Irvine, 1997, Benilova et al., 2012) we employed a second native separation method, asymmetric flow field-flow fractionation (AF4). AF4 is a flow-based method that does not require a stationary phase (Wahlund and Giddings, 1987) and therefore avoids some of the issues that may complicate interpretation of SEC. In AF4, separation takes place in a channel where sample retention is caused by the action of a cross-flow that is generated by a second independent stream that runs across the channel at right angles to the primary channel flow (Wahlund and Giddings, 1987, Wahlund and Litzen,

1989). The utility of AF4 was tested using the same synthetic A β monomer and dimer mixture as described above (Fig. 8A). Partial separation of monomer and dimer was achieved using AF4, with, the lower molecular weight monomer clearly eluting ahead of the heavier cross-linked dimer (Fig. 8C)*. Importantly, when GuHCl-treated AD TBS was analyzed by AF4, the brain-derived species fractionated in a manner similar to that seen with synthetic A β . Most monomer eluted in fx12 and fx13; and the majority of dimer eluted in fxs 13–14 (Compare Fig. 8C vs. Fig. 8D). These AF4 experiments agree with our SEC studies and provide additional evidence that authentic A β dimers are important constituents of Int mwt and High mwt A β .

In sum our results indicate that both authentic A β monomers and 7 kDa A β are present in high molecular weight assemblies and that the primary structure composition of 7kDa A β is distinct from that of monomers, with the former containing more N-terminal modifications and relevantly higher levels of A β 42.

Discussion

We previously reported that water-soluble extracts of AD brain potentially disrupt hippocampal long term potentiation (LTP), alter synaptic form and number, and impair memory consolidation (Shankar et al., 2008, Barry et al., 2011, Freir et al., 2011, Borlikova et al., 2013). In all cases these effects were shown to be dependent on A β and were reversed when A β was specifically removed (Shankar et al., 2008, Barry et al., 2011, Freir et al., 2011, Borlikova et al., 2013). The A β present in these samples migrated on SDS-PAGE with molecular weights consistent for monomers and dimers – a pattern we have seen in aqueous extracts of 33 AD brains used in this study and in more than 100 AD brains analyzed in our lab (Shankar et al., 2008, Mc Donald et al., 2010, Mc Donald, 2012) (J. Mc Donald, T. O'Malley, W. Liu, A. Mably and D. Walsh, unpublished). However, since the gels used to resolve these species are highly denaturing, the size estimates they provide do not necessarily reflect native A β assembly size. Rather, they simply indicate the presence of at least two different species.

Because fibrils are removed by centrifugation the A β species detected on SDS-PAGE are unlikely to be SDS-induced breakdown products of fibrils. Thus, the ~4 kDa species could be true monomers, and/or monomers derived from pre-fibrillar assemblies that are unstable when electrophoresed in SDS. Interpretation of the ~7 kDa band is more complicated. The most straightforward explanation for these species relies on SDS-PAGE providing an accurate estimate of molecular weight. In this case, the ~7 kDa material could be attributed to four different entities: (i) native dimers of A β formed from two A β monomers tightly bound together or covalently cross-linked, (ii) A β dimers that are generated from the denaturation of pre-fibrillar assemblies (O'Nuallain et al., 2010), (iii) small ~7 kDa fragments of APP that contain all or part of the A β sequence (Portelius et al., 2013, Kaneko et al., 2014, Welzel et al., 2014), or (iv) A β monomer bound to another biomolecule such that it alters its migration on SDS-PAGE, SEC and AF4. If the molecular weight predicted by denaturing gel electrophoresis is unreliable yet other explanations would be possible. For

*Footnote: on AF4, low molecular weight species elute first.

instance, certain N-terminal fragments of A β (e.g. A β 1-20) are known to migrate aberrantly on SDS-PAGE and produce bands with apparent molecular weights of ~6–7 kDa (Chesneau et al., 2000). Similarly, it is well-recognized that monomeric A β 1-42 can give rise to small amounts of anomalously migrating species (Hepler et al., 2006) which have been erroneously designated as “trimers” and “tetramers” (Chromy et al., 2003).

In this study we set out to shed light on the native assembly size of A β found in aqueous extracts of AD brain and to clarify the nature and involvement of the material that migrates on SDS-PAGE at ~7 kDa. Previously, we used native SEC to analyze A β in an aqueous extract from a single AD brain and we found that A β eluted in fractions consistent with monomer, dimer and high molecular weight species of undefined size (Shankar et al., 2008). Detection of a ~7 kDa species by SEC provided the first support that SDS-PAGE accurately reflected the molecular weight of at least some of the ~7 kDa species. Moreover, when each SEC fraction was tested for its ability to block LTP, only the dimer fraction had significant activity. Together these results demonstrate that the SEC-isolated ~7 kDa species is physically and functionally distinct from the ~4 kDa monomer. In the current study we used SEC to analyze water-soluble extracts from 33 AD brains. We found ~7 kDa A β species in a third of the samples, thus confirming that some of the ~7 kDa SDS-PAGE species were indeed native dimers and could be detected in the absence of monomer. Intermediate (~30–150 kDa) or high (~160–700 kDa) molecular weight species which broke down on SDS-PAGE to yield ~7 kDa A β were detected in all the brain extracts studied (Fig. 5, Supplementary Fig. 5, Table 4). Although we did not assess the activity of different SEC fractions, the fact that the majority of AD brains contain no ~7 kDa A β species, but had abundant Int mwt/High mwt species that broke down on SDS-PAGE to yield dimers, suggests that synaptotoxicity can be mediated by multiple species, of which dimers are likely to be important building blocks. Moreover, in future studies it will be important to determine how the different profiles of High mwt, Int mwt, ~7 kDa A β species and true monomer relate to clinicopathological stage, postmortem interval (PMI) and ApoE genotype.

To determine if the breakdown products of High mwt and Int mwt A β are real dimers or artefacts of SDS-PAGE, we first treated aqueous extracts from AD brains with strong denaturants and then analyzed them using SEC (Fig. 7). In each brain extract tested there was a significant shift in the elution pattern of the component A β species, with denaturants causing a loss of High mwt and Int mwt material and an increase in the amount of A β eluting as ~7 kDa A β species and true monomer. Importantly, when covalently cross-linked A β dimers were chromatographed on SEC these eluted in exactly the same fractions as the chaotrope-treated, brain-derived A β dimers. Furthermore, using a separate chromatographic technique, AF4, we also found that the SDS-PAGE ~7 kDa species eluted differently from A β monomer. Thus multiple lines of evidence indicate that the aqueous phase of AD brain contains 2 major forms of A β , a 6.1 – 7.9 kDa heterogeneous species centered at ~7 kDa, which most likely represents a mixture of highly stable N-terminally truncated A β dimers and a range of species from 3.5 – 4.5 kDa and centered at ~4 kDa, i.e. A β monomer beginning at Asp1, N-terminally truncated A β and p3. Since dimers are particularly abundant breakdown products of aggregates, it seems likely that dimers are either a

fundamental building block of aggregates, or that monomer-monomer interactions are stabilized in aggregates such that when aggregates breakdown they yield SDS-stable dimers.

Despite extensive efforts the precise primary structure of these putative dimers remains enigmatic. Using mass spectrometry we have identified more than 10 different primary structures in SEC-isolated monomer fractions, but as yet we have been unable to obtain useful spectra from SEC-isolated dimers (Supplementary Fig. 8). Although these efforts provide no information about the primary structure of the ~7 kDa species, they again demonstrate a physical difference between SEC-isolated monomers and dimers. Given that dimers have been refractory to mass spectrometric analysis we used a quantitative WB approach to gain insight into the molecular composition of dimers. For this we employed nine monoclonal antibodies that specifically recognize different epitopes within A β and 3 antibodies to a region of APP just N-terminal to the A β domain. In extracts from eight AD brains, the majority of the material present in the ~7 kDa bands was recognized by mAbs specific for the A β 42 C-terminus (21F12, HJ7.4) and the mid-region of A β (4G8, 266), and to a lesser extent the A β 40 C-terminus (2G3, HJ2). The anti-APP mAb 1G6 recognises an epitope just N-terminal of A β and readily detected a recombinant NTE-A β , but importantly 1G6 did not detect the ~7 kDa band found in aqueous extracts of AD brain. These findings indicate that the ~7 kDa band contains neither N-terminally extended, nor C-terminally truncated A β species.

Given our demonstration that the ~4 kDa band contains at least three different species, (two of which are N-terminally truncated) it is evident that many different types of dimers could be formed (Roberts et al., 2012). In this regard it is important to note that the ~7 kDa band seen on SDS-PAGE is more broad and fuzzy than the ~4 kDa monomer. Under native SEC conditions this ~7 kDa A β dimer species is a predominant component of both Int mwt and High mwt assemblies. Although, it is unclear whether the Int mwt and High mwt A β species are homo-oligomers of A β , or are complexes of A β bound to other proteins e.g. ApoE (Hashimoto et al., 2012, Takeda, 2013), our data suggest that preventing the formation and further aggregation of A β dimers may offer a novel target for the treatment of AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

aCSF artificial cerebrospinal fluid

AD	Alzheimer's disease
Aβ	amyloid β -protein
APP	amyloid precursor protein
BSA	bovine serum albumin
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethyleneglycoltetraacetic acid
ELISA	enzyme linked immunosorbant assay
fx	fraction
GuHCl	guanidinium hydrochloride
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IP	immunoprecipitation
LTP	long term potentiation
mAb	monoclonal antibody
mwt	molecular weight
NTE	N-terminally extended A β
oELISA	A β oligomer-specific ELISA
PAGE	polyacrylamide gel electrophoresis
PBS(T)	phosphate buffer saline (0.05% (v/v) Tween 20)
PrA	Protein A-sephrose
SDS	sodium dodecylsulfate
SEC	size exclusion chromatography
TBS(T)	tris buffer saline (0.05% (v/v) Tween 20)
WB	Western blot

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Fig. 1. Epitopes of the anti-Aβ and anti-APP antibodies used in this study

Line diagram of APP with the Aβ domain in red, and the single letter amino acid code of Aβ1-42. The reported binding epitopes for each antibody is indicated with a solid line under the corresponding amino acid sequence (see Table 1 for literature references). The location of the aspartate residue N-terminal of the Asp1 of Aβ is indicated by an arrow located at -31.

Fig. 2. Immunoprecipitation/Western blotting reveals the presence of ~4 and ~7 kDa A β species in the aqueous phase of AD brain

TBS extracts from four AD brains were IP'd with AW7 or pre-immune serum (PI) and WB'd with a combination of the A β 40 and A β 42 specific mAbs, 2G3 and 21F12. AD brain numbers and whether PI or AW7 antiserum was used for IP is indicated below each lane. Molecular weight markers are shown on the left. M (single arrow) denotes monomer and D (double arrow) refers to dimer. Non-specific indicates bands detected when PI was used are indicated by a solid black line. To allow comparison between blots, A β 1-40 (5, 10 and 45 ng) and A β 1-42 (5, 10 and 45 ng) were electrophoresed on each gel. The pattern shown is representative of more than 100 AD brains analyzed by this method, the sensitive of which is sufficient to detect A β at concentrations as a low as ~10 ng/g wet weight tissue.

Fig. 3. The primary structure composition of the ~4 and ~7 kDa species are different
 TBS extracts from four AD brains were IP'd with AW7 antiserum or pre-immune serum (PI) and WB'd with 3D6 (A), 6E10 (B), 4G8 (C), 2G3 (D) or 21F12 (E). To allow comparison between blots, Aβ1-40 (5, 10 and 45 ng) and Aβ1-42 (5, 10 and 45 ng) were electrophoresed on each gel. The epitope recognized by each mAb is indicated in Figure 1. AD brain numbers and use of PI or AW7 for IP is indicated below each lane. Molecular weight markers are shown on the left; M denotes monomer and D, dimer. Non-specific

bands detected when PI was used for IP are indicated by a solid black line on the right of each blot.

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Fig. 4. Aβ in AD-TBS extracts contains little pE3 Aβ and no detectable N-terminally extended Aβ species

TBS extracts from the same four AD brains shown in Figures 2 and 3 were IP'd with AW7 or pre-immune serum (PI) and WB'd with 1G6 (A) or mAb 07/1 (C). Recombinantly produced -31Aβ-40 (5, 10 and 45 ng) included on gels in panel A were readily detected, whereas no 1G6 immunoreactive species were detected in AD-TBS samples. Importantly, when the same blots were reprobed with a combination of 2G3 and 21F12 (B), abundant ~4 and ~7 kDa species were detected. AβpE3-40 (5, 10 and 45 ng) standards were readily detected with 07/1. A mAb 07/1-immunoreactive band that co-migrated with synthetic

A β pE3-40 was detected in one of the 4 AD brains examined. When the same blots were reprobed with a combination of 2G3 and 21F12 (D), abundant ~4 and ~7 kDa species were detected. AD brain numbers and the use of PI or AW7 for IP is indicated below each lane. Molecular weight markers are shown on the left. M denotes monomer and D, dimer. * indicates N-terminally extended (NTE) –31A β -40 standard.

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Fig. 5. Aqueous extracts of human brain contain up to 4 different sized A β assemblies separable by SEC

TBS extract (1 ml) from one AD brain (AD33) was chromatographed on a Superdex 75 10/30 HR column (A and B). Half ml fractions were collected, a 0.25 ml aliquot was lyophilized and analyzed by WB using a combination of 2G3 and 21F12 (A) or a monoclonal antibody, 46-4, which does not recognize A β (B). Bands specifically reactive with anti-A β mAbs were detected in fxs 1 and 2, (High mwt), 3, and 4 (Int mwt) 17 and 18 (monomer). A darker exposure (A inset) is shown to highlight bands in fxs 17 and 18. Two ml of AD-TBS (AD28) was chromatographed on a Superdex 75 10/30 HR column linked in

tandem with a Superdex 200 10/30 HR column (C). Fractions (1 ml) were lyophilized and analyzed by WB using 2G3/21F12. Because the fractions from a single chromatographic run couldn't be accommodated on a single gel, samples were electrophoresed on 2 (A and B) or 3 (C) gels alongside synthetic A β 1-42 standards (not shown) which were used to normalise the exposure of each blot. Species that migrated on SDS-PAGE as monomer and dimer are indicated by arrows and molecular weight markers shown on the left, and fraction numbers are provided below each lane. Four peaks of A β immunoreactivity were detected in the SEC fractions, and based on their elution relative to SEC standards are designated as (i) high molecular weight (high mwt), (ii) intermediate molecular weight (Int mwt), (iii) ~7 kDa A β species and (iv) true monomer (monomer) - these species are highlighted with an underline. The elution of linear dextran standards is indicated by up-pointing arrows and labelled 5, 12, 25, 80, 150, 270 kDa, while the elution of globular standards, is indicated by down-pointing arrows labelled 1, 17, 44, 158 and 670 kDa. Fraction 0 indicates the peak fraction in which Blue dextran eluted. Non-specific indicates bands detected when 46-4 was used.

AmAc**aCSF****oELISA** **A β x-42 ELISA**

Fig.6. High and Intermediate mwt oligomers are detected by ELISA in aqueous extracts of AD brain fractionated on SEC in physiological buffer

TBS extract (1 ml) from AD brain 30 was chromatographed on a Superdex 75 10/30 HR column eluted in 50 mM ammonium acetate (A and C) or artificial CSF (aCSF) (B and D). Half ml fractions were collected and analyzed using the oligomer-specific ELISA (oELISA) (A and B) or the A β x-42 ELISA (C and D). High/intermediate mwt oligomers were detected in fxs 1–4, whereas the majority of A β x-42 was found in fxs 14–18. Oligomer concentrations were calculated using an ADDL standard with units of equivalent weight of A β 1-42 monomer (see Yang et al. 2015) and A β x-42 concentration were calculated based on the molar content of SEC-isolated A β 1-42 monomer. Fraction “0” was not examined by ELISA, and ND indicates that the concentration was not determined.

Fig. 7. AD-TBS extract contains high molecular weight Aβ complexes which chaotropes break down to yield mostly SDS-stable dimer

An AD-TBS extract (AD31) was chromatographed on a Superdex 75 10/30 HR column (A). One ml fractions were collected, lyophilized and analyzed by WB using 2G3 and 21F12. The same AD-TBS (1 ml × 3) was IP'd with AW7, and the antigen-antibody complex eluted from the protein A sepharose beads by vortexing in 150 mM AmOH. The AmOH eluate was lyophilized, and then incubated in 6 M GuHCl (B), 88 % formic acid (C) or 2 M thiourea/6M urea (D) and chromatographed on a Superdex 75 10/30 HR column. One ml fractions were lyophilized and analyzed by WB using 2G3 and 21F12. Molecular weight

markers are shown on the left and fraction numbers are indicated below each lane. Exposures of the blots were adjusted so that the intensity of the synthetic A β 1-42 standards was comparable. Non-specific bands are indicated. Fraction 0 refers to the elution of Blue dextran. Bands that migrate with molecular weights consistent for A β monomer (M) and dimer (D) are indicated with arrows. The patterns shown are representative of that seen with aqueous extracts from 4 other AD brains.

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Fig. 8. AD-TBS A β treated with GuHCl elutes from SEC and AF4 predominantly as monomer and dimer

A mixture of A β 1-42 monomer and DiYA β 1-40 dimer was resuspended in 6 M GuHCl, boiled for 10 min and chromatographed on a Superdex 75 10/30 HR column. One ml fractions were collected, lyophilized and analyzed by WB using 2G3 and 21F12 (A). In a similar manner, AD-TBS (AD 21) was IP'd with AW7 and the antigen-antibody complex eluted from Protein A sepharose beads by boiling in 6M GuHCl and chromatographed (B). One ml fractions were collected and analyzed by WB using 2G3 and 21F12 (B). The same samples as used in (A) and (B) were also analyzed using AF4. Samples were injected onto a

24.6 cm long channel and 1 ml fractions were collected, lyophilized and analyzed by WB. Results for the synthetic A β mixture and the AD TBS extract are shown in (C) and (D), respectively. SDS-PAGE molecular weight markers are shown on the left, while elution of linear dextrans from SEC or AF4 are indicated by upward facing arrows. The elution of blue dextran is shown by an upward facing blue arrow for SEC (fx 0), but for AF4 blue dextran eluted outside the range shown (fx 29), a horizontal arrow denotes the later elution of blue dextran. No specific immunoreactive bands were detected above 10 kDa so blots were cropped accordingly. M refers to monomer and D to dimer.

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Fig. 9. SEC-isolated A β monomer and dimers have distinct primary structure compositions
 Five ml of AD TBS extract (AD43) was IP'd with AW7 (10×0.5 ml), and the antigen-antibody complex eluted from the protein A sepharose beads by vortexing in 150 mM ammonium hydroxide (AmOH). The AmOH eluate was lyophilized, and the lyophilizate incubated in 6 M GuHCl and then chromatographed on a Superdex 75 10/30 HR column. Half ml fractions were collected and 100 μ l of each fraction was lyophilized and analyzed by WB using 2G3 and 21F12 (A). The remaining fractions were pooled in groups of 3 as indicated on the figure. These pools were lyophilized, dissolved in sample buffer and a

sample of each pool (in duplicate) used for western blotting with 1G6 to detect N-terminally extended peptides and the same blot then serially reprobed with anti-A β mAbs in the following order: 3D6, 6E10, and a combination of 2G3 and 21F12 (B). Molecular weight markers are shown on the left and the SEC origins of the samples are indicated below each lane. Exposures of the blots were adjusted so that the intensity of the synthetic A β 1-40 standards are comparable. Non-specific indicates bands detected when 4G4 was used (not shown). Bands that migrate with molecular weights consistent for A β monomer (M) and dimer (D) are indicated with arrows.

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Table 1

Antibodies used in this study and their sources.

Antibody	Type	Recognises	Dilution for IP	Western blot conc.	ELISA conc.	Source/Reference
1G6	Monoclonal	APP 573–576	-	1 µg/ml	-	Covance/ (Halford and Russell, 2009)
Z31	Polyclonal	APP 577–596		1:500	-	Selkoe Lab/ (Shoji et al., 2000)
Pre-β	Polyclonal	APP 577–596		1:500	-	Selkoe Lab/ (Podlisny et al., 1995)
3D6	Monoclonal	Residue 1–5 of Aβ	-	1 µg/ml	1 µg/ml	Elan / (Johnson-Wood et al., 1997)
07/1	Monoclonal	Truncated Aβ that starts with pyroglutamate	-	2 µg/ml	-	Probiolog/ (Frost, 2013)
6E10	Monoclonal	Residues 6–10 of Aβ	-	1 µg/ml	-	Signet/ (McLaurin et al., 2002)
266	Monoclonal	Residues 16–23 of Aβ	-	1 µg/ml	3 µg/ml	Elan/ (Seubert et al., 1992)
4G8	Monoclonal	Residues 17–24 of Aβ	-	1 µg/ml	-	Signet/ (Portelius et al., 2006)
2G3	Monoclonal	Aβ terminating at Val40	-	1 µg/ml	-	Elan / (Johnson-Wood et al., 1997)
H12	Monoclonal	Aβ terminating at Val40	-	1 µg/ml	-	Holtzman D.M./ (Kim et al., 2012)
21F12	Monoclonal	Aβ terminating at Ile42	-	1 µg/ml	1 µg/ml	Elan / (Johnson-Wood et al., 1997)
H17.4	Monoclonal	Aβ terminating at Ile42	-	1 µg/ml	-	Holtzman D.M./ (Kim et al., 2012)
1C22	Monoclonal	Aβ aggregate specific	-	-	3 µg/ml	Walsh lab/ (Yang et al., 2015)
AW7	Polyclonal	Pan Aβ	1:80	-	-	Walsh Lab/ (Mc Donald et al., 2012)
46-4	Monoclonal	anti-HIV antibody, no Aβ reactivity (used as a negative control)	-	1 µg/ml	-	ATCC/ (Reeves et al., 1995)

Table 2

Demographic details of the cases used in this study.

Case	Source	Age	Gender	Neuropathology diagnosis	PMI (hr)	Clinical diagnosis
AD1	Asterand UK Ltd.	78	F	AD	5.33	AD
AD2	Washington University	80	M	AD	16	AD
AD3	UCLA	87	F	AD	75	NA
AD4	Beaumont Hospital, Dublin	55	F	AD	NA	AD
AD5	Beaumont Hospital, Dublin	66	F	AD	<48	AD
AD6	Beaumont Hospital, Dublin	73	F	AD	<24	AD
AD7	University College London	80	F	AD	32.3	AD
AD8	UCLA	96	F	AD	9	NA
AD9	UCLA	64	M	AD	34	AD
AD10	UCLA	70	M	AD	6	NA
AD11	UCLA	87	M	AD	5	NA
AD12	UCLA	81	M	AD	15	NA
AD13	Massachusetts General Hospital	83	F	AD	NA	AD
AD14	Massachusetts General Hospital	69	F	AD	16	AD
AD15	UCLA	84	F	AD	7	NA
AD16	UCLA	80	F	AD	3	NA
AD17	Washington University	79.6	F	AD	17.5	AD
AD18	Massachusetts General Hospital	69	F	AD	4.5	AD
AD19	Massachusetts General Hospital	74	F	AD	6	AD
AD20	University College London	51	F	AD	32.3	AD
AD21	UCLA	79	F	AD	5	AD
AD22	Massachusetts General Hospital	83	F	AD	<25	AD
AD23	Massachusetts General Hospital	81	F	AD	24	AD
AD24	Massachusetts General Hospital	80	NA	AD	NA	NA
AD25	Massachusetts General Hospital	69	F	AD	3	AD
AD26	University of Minnesota	84	NA	AD/Vas dementia	NA	AD/LBD

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Case	Source	Age	Gender	Neuropathology diagnosis	PMI (hr)	Clinical diagnosis
AD27	Washington University	80	F	AD	5.15	AD
AD28	UCLA	79	M	AD	NA	NA
AD29	Massachusetts General Hospital	75	F	AD	<48	AD
AD30	Massachusetts General Hospital	92	F	AD	4	AD
AD31	Massachusetts General Hospital	89	F	AD	4	AD
AD32	Massachusetts General Hospital	79	F	AD	NA	AD
AD33	Massachusetts General Hospital	74	F	AD	8	AD
C1	Massachusetts General Hospital	93	F	Control	5	Not demented
C2	Massachusetts General Hospital	44	F	Control	18	Not demented
C3	Massachusetts General Hospital	87	M	Control	48	NA

M refers to male, F denotes female, NA = information not available. PMI = Post-mortem interval

Table 3

Qualitative scoring of Aβ band intensities visualized by Western blotting.

		3D6	6E10	4G8	2G3	21F12
AD25	Mon1	++	+	++	+	++
	Mon2	-	++	++	+	++
	Mon3	-	-	-	-	++
	Dimer	+	+	+++	+	+++
AD31	Mon1	+	+	+	-	++
	Mon2	-	+	+	-	++
	Mon3	-	-	-	-	+
	Dimer	-	-	++	-	+++
AD18	Mon1	+	+	+	+	+
	Mon2	-	++	+	+	+
	Mon3	-	-	-	-	+
	Dimer	-	-	++	+	++
AD29	Mon1	++	++	++	+	++
	Mon2	-	++	++	++	++
	Mon3	-	-	-	-	++
	Dimer	+	+	+++	+	+++
AD30	Mon1	+	+	++	++	++
	Mon2	-	+++	++	++	++
	Mon3	-	-	-	-	+
	Dimer	-	+	+++	++	++
AD33	Mon1	++	++	++	++	++
	Mon2	-	++	++	++	++
	Mon3	-	-	-	-	++
	Dimer	-	+	+++	++	+++
AD32	Mon1	+	+	+++	+	++
	Mon2	-	++	+++	++	++
	Mon3	-	-	-	-	+

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		3D6	6E10	4G8	2G3	21F12
	<i>Dimer</i>	–	+	+++	++	+++
AD19	<i>Mon1</i>	+	+	+	+	+
	<i>Mon2</i>	–	++	+	+	++
	<i>Mon3</i>	–	–	–	–	+
	<i>Dimer</i>	–	–	++	++	++

For an example of all three monomer bands see Supplementary Fig. 1. Estimates of abundance were made by visual inspection of WBs shown in Fig. 3 and Supplementary Fig. 2 and are the average of determinations from 3 independent observers.

Not detected = –
Detected, < 5 ng = +
Detected, < 10 ng = ++
Detected between 10 ng and 45 ng = +++

Table 4

SEC analysis of AD brains.

Case #	High mwt	Intermediate mwt	~7 kDa A β species	True Monomer	Column
AD1	-	+ M +D	+	-	S75
AD2	+ M +D	-	-	+	S75
AD3	+ M +D	+ M +D	+	+	S75
AD4	-	+ D	+	+	S75
AD5	+ D	+ D	+	-	S75
AD6	+ D	+ D	-	+	S75
AD7	-	+ M +D	+	+	S75
AD8	-	+ M +D	-	-	S75
AD9	+ M +D	+ M +D	-	-	S75
AD10	-	+ M +D	-	-	S75
AD11	-	+ M +D	-	-	S75
AD12	-	+ M +D	-	-	S75
AD13	-	+ M +D	-	-	S75
AD14	-	+ M +D	-	-	S75
AD15	-	+ M +D	-	-	S75
AD16	-	+ M +D	-	-	S75
AD17	-	+ M +D	-	-	S75
AD18	+ M +D	+ D	+	+	S75
AD19	-	+ M +D	-	-	S75
AD20	-	+ D	+	+	S75
AD21	+ M +D	+ M +D	-	-	S75
AD22	-	+ M +D	-	-	S75
AD23	-	+ M +D	-	-	S75
AD24	-	+ M +D	-	-	S75
AD25	+ M +D	-	-	-	S75
AD26	+ D	+ M +D	+	-	S75/S200
AD27	+ M +D	+ D	+	+	S75/S200

Case #	High mw	Intermediate mw	~7 kDa Aβ species	True Monomer	Column
AD28	+ D	+ D	+	+	S75/S200
AD29	-	+ M + D	-	-	S75
AD30	+M	+M + D	-	-*	S75
AD31	-	+ M + D	-	-	S75
AD32	-	+ M + D	-	-	S75
AD33	-	+ M + D	-	+	S75

The presence or absence of a species is indicated by + or -, respectively. M and D refers to the migration of Aβ on SDS-PAGE, were M=monomer, D=dimer. +* = a very weak signal by WB, but readily detected by ELISA, and -* not detectable by WB, but detectable by ELISA.