

AD synapses contain abundant A β monomer and multiple soluble oligomers, including a 56-kDa assembly

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Abstract

Much evidence indicates that soluble amyloid beta (A β) oligomers are key mediators of early cognitive loss, but the localization and key peptide species remain unclear. We have used flow cytometry analysis to demonstrate that surviving Alzheimer's disease (AD) synapses accumulate both A β and phosphorylated tau (p-tau). The present experiments use peptide-specific X-map assays and Western blot analyses to identify the A β peptide species in synaptosome-enriched samples from normal human subjects, neurologic controls, and AD cases. A β 40 peptide levels did not vary, but both A β 42 and A β oligomers were increased in soluble AD extracts, with oligomer levels 20-fold higher in aqueous compared with detergent extracts. In Western blot analysis, a ladder of sodium dodecyl sulfate (SDS)-stable oligomers was observed in AD cases, varying in size from monomer, the major peptide observed, to larger assemblies up to about 200 kDa and larger. Multiple oligomers, including monomer, small oligomers, a 56-kDa assembly, and amyloid precursor protein (APP) were correlated with the A β level measured in flow cytometry-purified synaptosomes. These results suggest that multiple amyloid precursor protein processing pathways are active in AD synapses and multiple soluble oligomeric assemblies may contribute to synaptic dysfunction.

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1. Introduction

A number of studies have documented that levels of soluble amyloid beta (A β) peptides are superior to amyloid deposits as correlates of cognitive decline in Alzheimer's disease (AD; McLean et al., 1999; Näslund et al., 2000). Accordingly, the original amyloid cascade hypothesis has evolved to propose that soluble oligomeric A β assemblies precede deposition and are the proximal cause of synaptic dysfunction

and early impairment in AD (see Walsh and Selkoe, 2007 for review). However, the size of the key assembly state and relevant downstream pathways remain the subject of intense study. Among natural low-n assemblies, dimers and/or trimers in particular have been isolated from AD brain and shown to impair cognition in vitro (Cleary et al., 2005; Klyubin et al., 2008; Shankar et al., 2008; Townsend et al., 2006). In the Tg2576 mouse model a larger assembly, (A β *56), possibly a multimer of smaller oligomers, was associated with cognitive decline in Tg2576 mice (Lesné et al., 2006).

Soluble A β peptides are associated with synaptic loss (Lue et al., 1999), and multiple studies have shown that

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soluble oligomers bind to dendritic spines in primary cultures (Lacor et al., 2004, 2007). Recent evidence also suggests that brief passive immunotherapy has acute and extended benefits on synaptic density and plasticity (Rozkalne et al., 2009; Spires-Jones et al., 2009). Consistent with synaptic A β release, interstitial A β levels are increased by synaptic activity (Cirrito et al., 2005, 2008), and have been shown to correlate with neurological status in patients with brain injury (Brody et al., 2008). Reasoning that study of surviving synaptic terminals is critical for understanding the sources for synaptic A β production and release as well as pathways leading to loss of synapses, we have analyzed human synaptosomal preparations by flow cytometry analysis and have shown that A β accumulates in synaptic terminals in multiple regions of AD brain. Phosphorylated tau (p-tau) also accumulates in A β -bearing synapses, and the colocalization of A β and p-tau is accompanied by increased synaptosome size, modest losses of the postsynaptic density scaffold protein, PSD-95, and increased cholesterol and ganglioside GM1 (Gyls et al., 2004b, 2007, 2008). With flow cytometry, the synaptosomal A β signal is best detected by an N-terminal antibody (10G4) that does not discriminate between peptides; the present study correlates the flow cytometry signal with a series of peptide and conformation-specific antibodies along with a series of A β peptide-specific assays on the Luminex platform. We report here that monomeric A β is prominent among multiple sodium dodecyl sulfate (SDS)-stable soluble A β species, including a 56-kDa assembly, in synaptic terminals from AD cortex.

2. Methods

2.1. Materials

The monoclonal anti-A β antibody 10G4 has been described previously (Mak et al., 1994). Polystyrene microsphere size standards were purchased from Polysciences, Inc. (Warrington, PA, USA), and rhodamine-conjugated anti-mouse antibody from Chemicon (San Diego, CA, USA). The following monoclonal antibodies were purchased: anti-SNAP-25 (Sternberger Monoclonals, Inc., Lutherville, MD, USA), anti-PSD 95 (Upstate Biotechnology, Lake Placid, NY, USA), 6E10 antibody (Signet Labs, Dedham, MA, USA), anti-synaptophysin from Abcam (Cambridge, MA, USA), 4G8 antibody (Covance, Denver, PA, USA), and anti-amyloid precursor protein (APP) 3E9 (MBL, Naka-ku Nagoya, Japan). A11 was the kind gift of C. Glabe (UC, Irvine, CA, USA), and OC antibody was received from R. Kaye (UTMB, Galveston, TX, USA). The rabbit anti-A β 42 and anti-A β -40 antibodies were from T. Golde (Mayo Clinic, Jacksonville, FL, USA).

2.2. Human brain specimens

Brain samples, primarily superior parietal (A7) cortex were obtained at autopsy from the Alzheimer's Disease Research Centers at USC and UCLA; for some experiments frontal (A9)

or parietal (A39) samples were substituted. Samples were obtained from a total of 14 cases (10 female, 4 male); 7 were diagnosed clinically and histopathologically with AD, and 3 were neurological control cases. The control cases included 2 Parkinson's disease (PD) and 1 tauopathy case. The 4 cognitively normal aged controls were confirmed histopathologically. The mean age of AD cases was 86.3, and 84.6 for normal and control cases. The mean postmortem interval for AD cases was 8.2 hours, and for normal and control cases was 7.0 hours.

2.3. P-2 preparation

Samples (~0.3–5 g), were minced and slowly frozen on the day of autopsy in 10% dimethyl sulfoxide and 0.32 M sucrose and stored at -70°C until homogenization. The crude synaptosome (P-2) fraction was prepared as described previously (Gyls et al., 2003), briefly, the homogenate was first centrifuged at 1000g for 10 minutes; the resulting supernatant was centrifuged at 10,000g for 20 minutes to obtain the crude synaptosomal pellet. Aliquots of P-2 are routinely cryopreserved in 0.32 M sucrose and banked at -70°C until the day of the experiment.

2.4. Immunolabeling of P-2 fraction

P-2 aliquots were immunolabeled for flow cytometry analysis according to a method for staining of intracellular antigens (Schmid et al., 1991). Pellets were fixed in 0.25% buffered paraformaldehyde (1 hour, 4°C) and permeabilized in 0.2% Tween 20/phosphate-buffered saline (PBS) (15 minutes, 37°C). Antibodies were labeled directly with Alexa Fluor 488 or 647 reagents according to kit directions (Zenon Alexa Fluor Labeling Kit, Invitrogen, Carlsbad, CA, USA). The labeled antibody mixture was added to P-2 aliquots (5 μL of P-2 pellet/sample, ~5–8 $\mu\text{g}/\mu\text{L}$) and incubated at room temperature for 30 minutes. Pellets were washed 2 times with 1 mL 0.2% Tween 20/PBS, and resuspended in PBS buffer (0.75 mL) for flow cytometry analysis. The synaptosomal pellet was dispersed for all washes and for incubations with fixative, detergent, and antibody, then collected by centrifugation (1310g at 4°C).

2.5. Flow cytometry

Data were acquired using the BD-FACSCalibur and the BD SORP LSRII analytic flow cytometers (Becton-Dickinson, San Jose, CA, USA) equipped with argon 488 nm, helium-neon 635 nm, and helium-cadmium 325-nm lasers. Five thousand particles were collected and analyzed for each sample. Debris was excluded by establishing a size threshold set on forward light scatter. Alexa 488 and Alexa 647 fluorochromes were detected by the FL1 and Ssc-W photomultiplier tube detectors, respectively. Analysis was performed using FCS Express software Version 3 (DeNovo Software, Ontario, Canada).

2.6. Synaptosome extracts

Crude P-2 fractions ($\times \mu\text{L}$ P-2 pellet, $\sim \mu\text{g}$ protein) were first extracted by sonication in a detergent-free buffer (10 mM Tris, 1 mM ethylene glycol tetraacetic acid (EGTA), 10% sucrose, pH 7.5) and then spun at 25,000g. The supernatant of this detergent-free extraction was used to quantify the levels of aqueous soluble A β species. The remaining pellet was extracted by sonication in the same buffer containing 1% N-lauroylsarkosyl (NLS) and spun at 300,000g. This supernatant was analyzed as the detergent soluble fraction.

2.7. Luminex assay

The aqueous and detergent soluble fractions were quantified using specific bead-based kits for aggregated A β , A β 40, and A β 42 (Invitrogen, CA, USA). Assays were performed on a Luminex instrument using X-map Technology and xPONENT Version 3 software (Luminex Corp, Austin, TX, USA). Standard curves were constructed from authentic standards included with each kit; the lower limit of quantification was 52 pg/mL for aggregated A β , and 50 pg/mL for A β 40 and A β 42. Each extract was analyzed in duplicate for each analyte.

2.8. Statistics

Student *t* tests were calculated using the Vassarstat interactive statistical web site (faculty.vassar.edu/lowry/VassarStats.html; Richard Lowry, Poughkeepsie, NY, USA). Correlation coefficients were calculated with the Spearman correlation coefficient procedure.

2.9. Western blot analysis

Samples (20 μg P-2 sample/lane) were boiled in Laemmli loading buffer (2% SDS, Invitrogen) and electrophoresed on 4%–20% Tris-Tricine gradient gels, then transferred to polyvinylidene fluoride membranes. Because actin and similar genes may be affected in AD samples, membranes were stained with Ponceau (immediately after transfer) and Coomassie blue (after immunolabeling) to ensure equal protein loading. Only blots with equal loading were quantified and included in figures. Membranes were blocked for 1 hour at room temperature in 10% nonfat dried milk in PBS, followed by incubation overnight at 4 °C with primary antibodies (1:1000) in PBS containing 0.05% Tween 20 (PBS-T) and 1.5% (wt/vol) albumin. After rinsing in PBS-T, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse (1:50,000) or anti-rabbit antibodies (1:100,000) in PBS-T with 1.5% albumin for 1 hour. Immunolabeled proteins were visualized by enhanced chemiluminescence (ECL) detection reagents (SuperSignal West Femto, Thermo Scientific, Rockford, IL, USA). Resulting films were scanned and quantified on a UVP BioSpectrum 600 imaging system using VisionWorks Version 6.6A software (Upland, CA, USA).

3. Results

3.1. Elevated A β in intact AD synaptosomes is accompanied by high levels of APP

Synaptosomes are resealed synaptic boutons that form when fresh tissue is gently homogenized with the appropriate shear force in isotonic sucrose. The preparation has been thoroughly characterized over several decades; typical synaptosomes contain presynaptic cytoplasm, synaptic vesicles, mitochondria, and cytoskeleton proteins enclosed by a spherical membrane, along with attached fragments of post-synaptic membrane and postsynaptic densities (see Dunkley et al., 2008 for review). Flow cytometry analysis of synaptosomes enables focus on a large and highly pure population (3000–5000 analyzed per case) of human synaptic terminals; by using an analysis gate to include only particles between 0.75 and 1.5 μm , we have previously shown the synaptosomal population in flow cytometry studies to be \sim 95% pure. This compares with approximately 80% for a gradient-purified synaptosomal preparation (Fein et al., 2008; Gyls et al., 2004a, 2007; Wolf and Kapatos, 1989). Synaptosomal purity for the present experiments is illustrated by SNAP-25 immunolabeling of a representative parietal cortex sample (Fig. 1A), compared with background (Fig. 1B).

Flow cytometry analysis of parietal cortex synaptosomes was used to compare A β labeling (with the antibody 10G4, an N-terminal antibody directed against residues 5–17), with labeling for APP (3E9 antibody; Fig. 1C and D). Marked synaptic A β accumulation is observed in AD parietal cortex, with little A β detected in cognitively normal aged control cases. APP is present at high levels in synapses, with approximately 65%–70% of synaptosomes positive for APP in AD and in controls (Fig. 1E and F). In aggregate, the fraction of A β -positive synaptosomes increases from 22% (\pm 8.2, n = 5) in age-matched control subjects to 55% in AD parietal cortex (\pm 4.6, n = 19; p < 0.001); however, the fraction of synaptosomes positive for APP in controls (65%; \pm 4.9, n = 5) was unchanged in surviving terminals in AD (65%; \pm 3.9, n = 19).

Sixty-four percent of synaptic terminals were labeled for A β with the 10G4 antibody in a representative PD case (Fig. 2A), and an even larger fraction (78%) was A β -positive in a PD case with 2 years of dementia, suggesting that synaptic A β elevations are not limited to AD.

With flow cytometry, bright specific immunolabeling is obtained with the antibody 10G4 in intact AD synaptosomes that are fixed and permeabilized for labeling of intracellular antigens (Fig. 1D and G). However, an A β 42-specific C-terminal antibody labels a positive fraction just above background, indicating that the C-terminal epitope is not exposed in situ (Fig. 2A). The N-terminal antibody 6E10, which is directed against a similar epitope to 10G4, likewise labels a much smaller fraction of synaptosomes in the same case (Fig. 2B). No immunolabeling is observed in intact

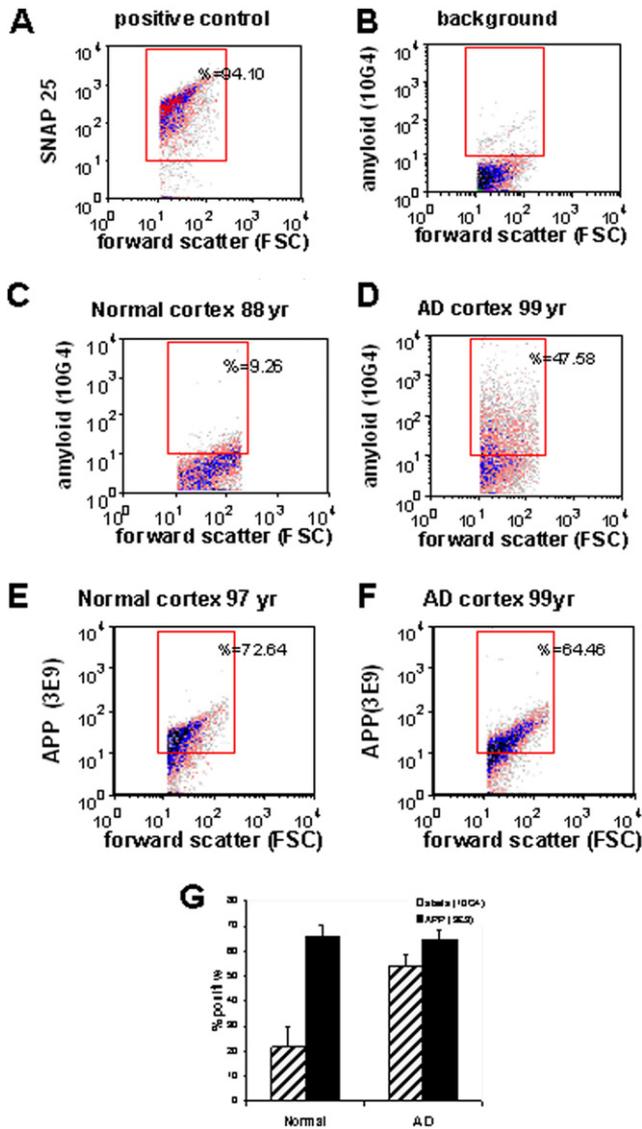


Fig. 1. Flow cytometry analysis of amyloid beta ($A\beta$) and amyloid precursor protein (APP) in Alzheimer's disease (AD) synaptosome. Representative flow cytometry controls are shown for: (A) an AD parietal cortex sample labeled for SNAP-25 as a positive control and indicator of synaptosomal purity, and (B) background labeling in the presence of a nonspecific isotype control. Representative synaptosomal $A\beta$ labeling is shown for a parietal cortex sample from an aged normal control case (C), and an AD case (D). Synaptosomal APP labeling is shown for a parietal cortex sample from an aged normal control case (E), and from an AD case (F). (G) Size of positive fraction for APP and $A\beta$ in aged cognitively normal ($n = 5$) and AD cases ($n = 19$); data were collected from 5000 terminals for each sample ($p < 0.001$, Student t test).

synaptosomes for the N-terminal conformation-specific antibodies A11 and OC, which detect prefibrillar and fibrillar oligomers respectively (not shown). Taken together, these results indicate limited flow cytometric detection of $A\beta$ peptide-specific epitopes in intact synaptosomes. This conclusion is in line with the observation that antigen masking has historically limited $A\beta$ detection in situ, and is a factor

contributing to false-negative results for intraneuronal localization of $A\beta$ (Gouras et al., 2005).

3.2. Soluble oligomers and $A\beta_{42}$ are the primary peptides in aqueous extractions

We next measured $A\beta$ with a number of biochemical assays to confirm and extend the flow cytometry observations of synaptic $A\beta$ accumulation. The goal was to compare $A\beta$ pools and peptides in a series of AD and normal cases; synaptosome-enriched P-2 fractions (crude synaptosomes; SEF) were used in order to obtain sufficient volume for the analyses. Western blot analyses were used to demonstrate the enrichment of pre- and postsynaptic elements in the P-2 compared with the initial sucrose homogenate and the initial low-speed pellet (P-1), which contains nuclei and large membrane fragments (Fig. 3A and B). Synaptophysin,

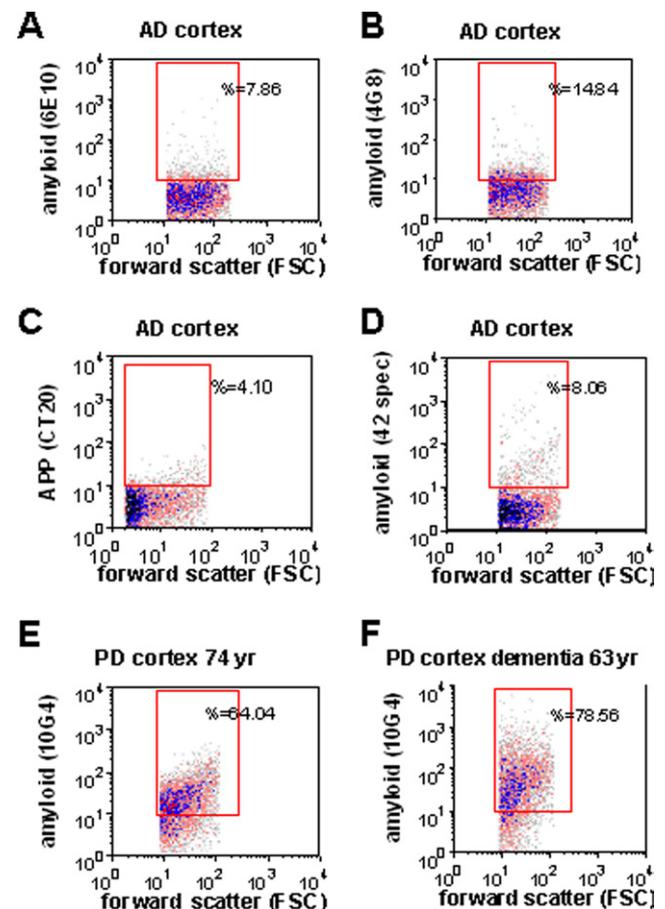


Fig. 2. Flow cytometry analysis of N-terminal and C-terminal antibodies; amyloid beta ($A\beta$) in Parkinson's disease (PD) and mixed dementia. (A–D) Representative samples of Alzheimer's disease (AD) cortex showing minimal immunolabeling in intact synaptosomes for the N-terminal antibodies 6E10 (A), 4G8 (B), CT20, a C-terminal amyloid precursor protein (APP) antibody (C), and an $A\beta_{42}$ -specific antibody (D). Synaptosomal $A\beta$ immunolabeling for a parietal cortex sample from a Parkinson's disease case without dementia (E; case information in Table 1, lane 5), and for a dementia case with a 2-year history of dementia caused by Parkinson's disease (F; case information in Table 1, lane 6).

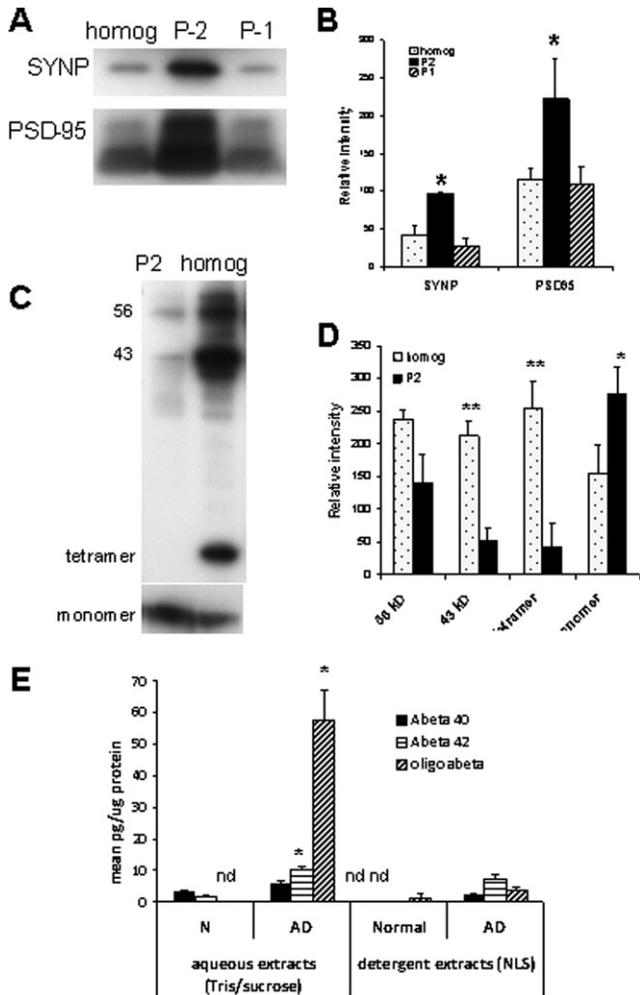


Fig. 3. Biochemical analysis of amyloid beta ($A\beta$)42, $A\beta$ 42 and oligomeric $A\beta$ in synaptosome-enriched fractions (P-2). (A) Western blots comparing levels of presynaptic (synaptophysin, SYN) and postsynaptic (PSD-95) markers in the initial homogenate, initial nuclear fraction (P-1) and synaptosome-enriched (crude synaptosome; P-2), with quantification illustrated in (B); $n = 4$, ** $p < 0.01$, * $p < 0.02$ compared with initial homogenate). (C) Western blot showing $A\beta$ level in P-2 compared with homogenate, with quantification in (D); ** $p < 0.01$, * $p < 0.05$ compared with initial homogenate). (E) Bead-based luminex biochemical assay for aqueous and detergent (1% N-laurylsarcosyl) extracts of synaptosome-enriched fractions from normal ($n = 2$) and Alzheimer's disease (AD) cases ($n = 7$). * $p < 0.05$ for AD versus normal (N) and for aqueous versus detergent-soluble extracts. nd, not detectable.

a synaptic vesicle protein involved in docking of presynaptic vesicles prior to exocytosis (Südhof, 1995), was increased in the P-2 compared with the initial homogenate by 2.3-fold ($p < 0.01$); P-2 enrichment for the postsynaptic N-methyl-D-aspartate scaffold protein PSD-95 (Kornau et al., 1995) was 1.92-fold (Fig. 3B; $p < 0.05$). Differential distribution of SDS-stable $A\beta$ peptide assemblies between the P-2 and initial homogenate is shown in Fig. 3C and D. Higher levels of 2 $A\beta$ oligomers (43 kDa and tetramer) in the initial homogenate compared with the P-2 fraction ($A\beta$ -42-specific antibody), with $A\beta$ monomer levels higher in

the P-2 (6E10 antibody). Increased monomer may result from breakdown of higher order oligomers during the Western blot analysis procedure, and seems likely to indicate impaired $A\beta$ clearance in synaptic terminals.

To determine the specific $A\beta$ peptide(s) in synaptosomes, we used specific bead-based immunoassays using the X-map technology (Luminex) to quantify 3 $A\beta$ species ($A\beta$ 40, $A\beta$ 42, and $A\beta$ oligomers of 40 or 42 amino acids) in sequential extractions of P-2 fractions prepared from fresh unfixed AD samples. To measure $A\beta$ in soluble and detergent fractions, P-2 pellets were first extracted by sonication in detergent-free buffer (Tris/EGTA/sucrose); the supernatant after centrifugation was used for the soluble extracts. The pellet was sonicated in the same buffer plus 1% N-laurylsarcosyl, centrifuged, and the supernatant used for the detergent extracts. Fig. 3E shows that $A\beta$ 40 levels did not differ between groups, but $A\beta$ 42 was higher in aqueous versus detergent extracts in AD samples (10.35 ± 0.74 vs. 7.38 ± 1.23 $\mu\text{g}/\mu\text{g}$; $p < 0.05$; $n = 7$ aqueous samples and 4 detergent samples). $A\beta$ 42 was also elevated in AD compared with control samples in aqueous (10.35 ± 0.74 vs. 1.69 ± 0.15 $\mu\text{g}/\mu\text{g}$; $p < 0.05$) extracts; $A\beta$ 42 was undetectable in detergent extracts from control cases ($n = 2$). The small number of controls was prompted by the large tissue volume required for extraction experiments, together with the limited availability of aged control cases and previous results showing relatively little synaptic $A\beta$ pathology in control cases (Gylys et al., 2007, 2004b). The aggregated $A\beta$ assay is a bead-based sandwich assay that uses the same antibody for capture and detection, therefore only proteins with multiple copies of the $A\beta$ N-terminus are quantified. Aggregated $A\beta$ levels were undetectable in control samples, but were 20-fold higher in aqueous compared with detergent extracts in AD samples (57.57 ± 9.51 vs. 2.62 ± 3.71 $\mu\text{g}/\mu\text{g}$; $p < 0.05$). Taken together, the Luminex assays indicate that soluble $A\beta$ is primarily $A\beta$ 42 peptide, with the majority in oligomeric ($n \geq 2$) form. Because assays using the same antibody for capture and detection cannot distinguish between multiple monomers bound to a carrier such as receptors for advance glycation endproducts (RAGE) or apolipoprotein E (apoE), the measure of oligomers may include carrier-bound $A\beta$.

3.3. $A\beta$ monomer and a 56 kDa SDS-stable peptide assembly are prominent among multiple $A\beta$ species

In order to gain size information about SDS-stable $A\beta$ assemblies in synaptosome-enriched fractions, Western blotting experiments were performed with a series of anti- $A\beta$ antibodies. For the present study we examined synaptosomes from a series of AD ($n = 7$) and normal control cases ($n = 4$). The series included 3 additional neurologic control cases: 2 with PD, and a third tauopathy dementia case that showed marked tau-immunoreactive neurofibrillary tangles and neuropil threads but no diffuse or neuritic plaques. Neurologic controls were included to better understand mixed pathol-

Table 1
Case information for synaptosome samples

Lane	Sex	Age	PMI (h)	Braak score	Frontal cortex atrophy	Neuritic plaques	Neurofibrillary changes (neuropil threads)	Amyloid fluorescence (RFU)	P-tau fluorescence (RFU)
Normal cases									
1	F	97	5.5	—	Mild	0	0	19.73	23.89
2	M	93	8.5	—	Mild	0	0	45.16	25.27
3	M	82	14.9	Normal with hippocampal sclerosis	Moderate	0	0	27.36	26.24
4	F	105	9	—	Moderate	0	0	23.25	17.19
PD cases									
5	M	74	7.8	PD	Mild	0	0	50.35	33.80
6	M	63	3.5	PD vs. Lewy body AD 2 y dementia	Moderate	Moderate	Sparse	66.50	25.18
Tauopathy									
7	F	72	5	Dementia	Severe	0	Frequent	43.99	140.96
AD cases									
8	F	89	12	IV–V; 10 y dementia	Mild	Moderate	Sparse	79.23	43.07
9	F	87	10	V–VI; 5 y dementia	Mild	Frequent	Mild	145.79	79.84
10	F	80	11	VI; 7 y dementia	Moderate	Moderate	Prominent	77.11	23.99
11	F	86	5	VI; 11 y dementia	Severe	Moderate	Moderate	104.92	58.70
12	M	82	5	VI; mixed AD/Lewy body AD	Severe	Sparse	Sparse	82.71	41.40
13	M	83	7	V; 2 y dementia	Mild	Frequent	Moderate	290.95	94.80
14	F	99	11.5	V; 2 y dementia	Moderate	Sparse	0	74.85	45.64

Cases used for Western blot analysis. Neuropil threads and plaques are reported for frontal cortex and refer to silver (Gallyas) stain. The plaque number includes plaques with and without cores; sparse (< 5 per field), moderate (6–20 per field), frequent (21–30 per field). AD, Alzheimer's disease; F, female; M, male; PD, Parkinson's disease; PMI, post mortem interval; p-tau, phosphorylated tau; RFU, mean relative fluorescence for amyloid beta ($A\beta$) and p-tau antibody fluorescence measured by flow cytometry (mean in superior parietal A7 cortex).

ogy and the disease specificity of synaptic pathology in AD; for example, 1 of the PD cases displayed mixed pathology with a 2-year history of dementia, and a diagnosis of Lewy body AD was considered for this case. Case information and synaptic $A\beta$ levels measured by flow cytometry are presented for each case in Table 1 along with the lane number for each case in Western blot analysis.

In most AD cases, a ladder of SDS-stable oligomers was detected with multiple antibodies, varying in size from monomer to larger assemblies up to about 200 kDa. The N-terminal antibodies 6E10 and 10G4 are directed against similar $A\beta$ epitopes (6E10 against residues 5–10 and 10G4 against 5–17), but they differentially detect oligomers and APP (Fig. 4A and B). The antibody 10G4 labels primarily tetramers, oligomers in the range of 40–48 kDa; 10G4 also preferentially detected large aggregates. The 6E10 antibody (Fig. 4B) preferentially detected APP and small to midrange oligomers including a 56-kDa species. With both antibodies, strong monomer bands were detected in all AD cases. In the same cases, an antibody specific for $A\beta_{42}$ peptide (Fig. 4C), prominently labeled a 56-kDa dodecamer ($A\beta$ -star) among a ladder of oligomers. The 56-kDa assembly was not detected by an antibody directed against $A\beta_{40}$, which, like 10G4, preferentially detected the 43-kDa assembly (Fig. 4D). Quantification of AD-selective $A\beta$ peptides is shown in Fig. 4E; monomer and 3 $A\beta$ assemblies (large aggregates, 56 kDa and 36 kDa), along with APP and the C-terminal fragment were significantly increased in AD versus normal synaptosome-enriched samples. Increased APP in

AD immunoblots but not flow labeling of intact synaptosomes suggests localization to an interior compartment, possibly endosomal and/or lysosomal.

The tauopathy case was positive for APP with several antibodies (22c11, 3E9, CT20, not shown), but $A\beta$ assemblies were poorly detected in multiple experiments. In terms of $A\beta$, both PD cases demonstrated a ladder of midrange to large oligomers with the 42-specific antibody. The PD case with dementia (720, lane 6) had a differential diagnosis of AD, Lewy body variant with a final diagnosis of PD; in most experiments this case demonstrated prominent $A\beta$ monomer and aggregates that were similar to the pathology observed in the AD cases.

3.4. Monomers, APP, and multiple $A\beta$ oligomers correlate with synaptic $A\beta$ measured by flow cytometry in size-purified synaptosomes

In contrast to measures of $A\beta$ by size-gated flow cytometry in a population of synaptosomes that is approximately 95% pure, the P-2 fractions used for Western blot analyses are enriched in synaptosomes, but also contain free mitochondria and vesicles, myelin, and membrane fragments in addition to intact synaptosomes. Therefore Western blot analyses results were correlated with the synaptic $A\beta$ level for each case measured by flow cytometry analysis of P-2 fractions with the 10G4 antibody (Table 1). A number of $A\beta$ -related peptides and assemblies from the Western blot

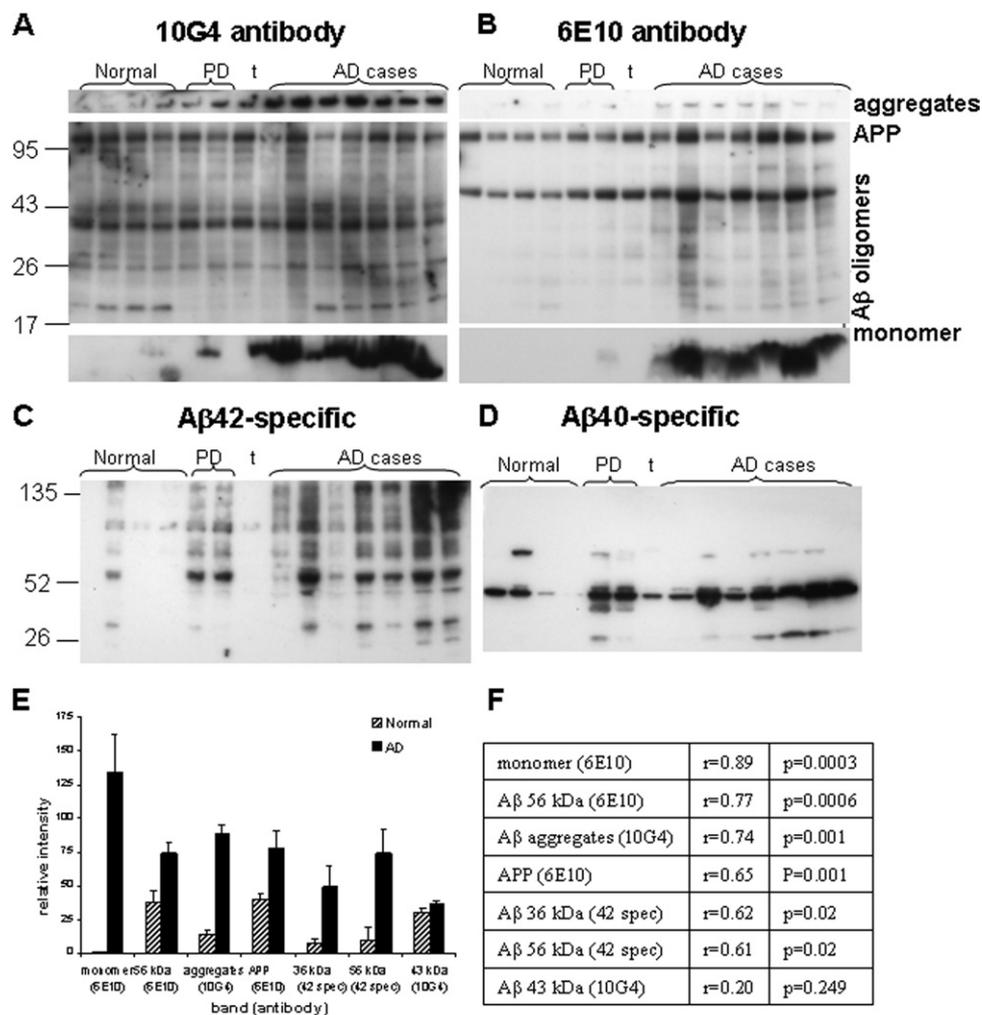


Fig. 4. Anti-amyloid beta ($A\beta$) antibody Western blots in synaptosome-enriched fractions. Western blots are shown for a series of normal ($n = 4$) and Alzheimer's disease (AD) cases ($n = 7$). Controls include 2 PD cases and 1 tauopathy case (t); the right PD case (lane 6) had a 2-year history of dementia. Case information is presented in Table 1. Anti- $A\beta$ antibodies illustrated are the N-terminal antibodies 10G4 (A), and 6E10 (B), and for C-terminal antibodies directed at $A\beta$ 42 (C), and at $A\beta$ 40 (D). (E) Quantification of AD-selective peptide assemblies from Western blots; $p < 0.05$ for all comparisons except the 43-kDa band. (F) Correlation of Western blot $A\beta$ peptides with flow cytometry $A\beta$ fluorescence in size-purified synaptosomes (see Table 1, amyloid fluorescence column).

series correlated significantly with the flow cytometry measure of $A\beta$ fluorescence (Fig. 4F), with the highest correlation observed for $A\beta$ monomer. Other assemblies with significant correlations included APP, aggregates, and SDS-stable assemblies at 36 kDa and 56 kDa.

The conformation dependent antibody A11, selective for prefibrillar oligomers, also labeled a major assembly at about 43 kDa, along with strong trimer/tetramer bands at 12 and 16 kDa in 3 of the 7 AD cases (Fig. 5A). Interestingly, the trimer/tetramer assembly was present in both PD cases, and was most prominent in the nondemented PD case (711, left-most PD lane). However, these may also represent alpha synuclein or other oligomers, as A11 sensitivity to oligomers is not limited to $A\beta$ oligomers (Kayed et al., 2003). Fibrillar oligomers were detected by the OC antibody in P-2 fractions as a series of SDS-stable oligomers

between 26 and 56 kDa in size (Fig. 5B); bands at approximately 42, 45, and 51 kDa were the most specific for AD. Fibrillar oligomers are conformationally distinct from prefibrillar oligomers (Kayed et al., 2007; Tomic et al., 2009) and were also prominent in the nondemented Parkinson's case. The widely used 4G8 antibody (directed against $A\beta$ residues 17–24; Fig. 5C), in contrast to other N-terminal antibodies, did not sensitively detect oligomers and primarily labeled a major band at approximately 43 kDa; this band was also observed with the 10G4 and 40-specific antibodies and was seen in normal as well as AD cases (Fig. 4A and D). A 43-kDa assembly is consistent with an $A\beta$ nonomer as described for in vitro $A\beta$ assemblies (Bitan et al., 2003); this assembly is also prominent in Western blots immunolabeled for apoE and may represent an $A\beta$ dimer bound to apoE (Permanne et al., 1997).

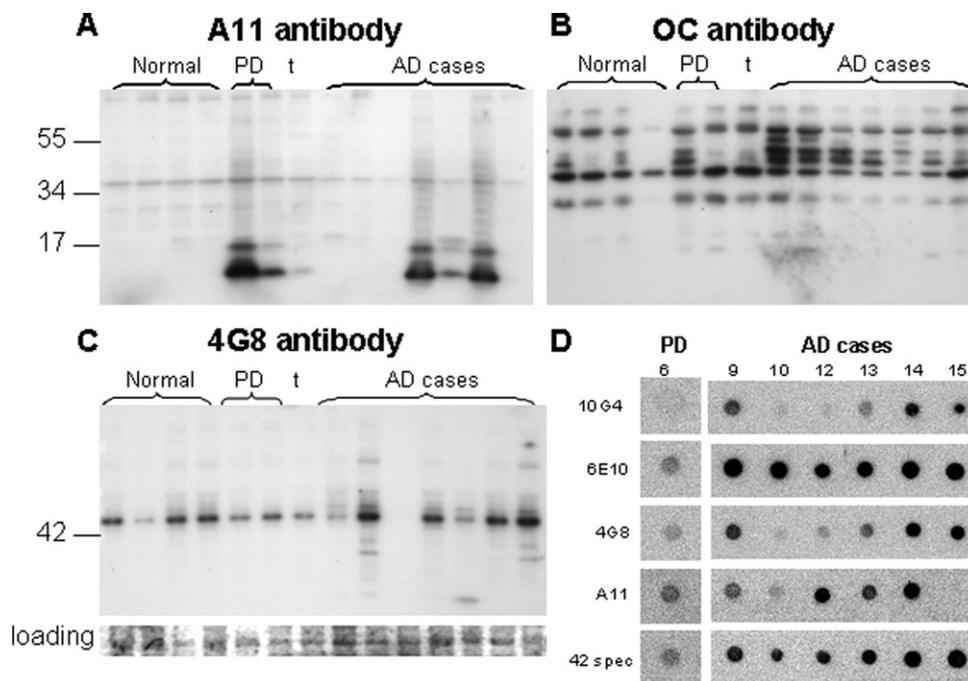


Fig. 5. Amyloid beta ($A\beta$) peptide species conformation. Western blots labeled with conformation-selective antibodies in the same normal ($n = 4$) and Alzheimer's disease (AD) cases ($n = 7$) used for Fig. 3. (A) Prefibrillar oligomers labeled with A11; (B) fibrillar oligomers labeled with the antibody OC. (C) Western blot for the N-terminal $A\beta$ antibody 4G8 with Coomassie blue-stained membrane as loading control. (D) Dot blots using detergent-free sonicated synaptosome-enriched fractions; cases shown correspond to the lane number in (A) and (C), i.e., the Parkinson's disease (PD) case is the right-most case in lane 6 of Western blots.

Most protein complexes and oligomers dissociate when treated with SDS, but for amphipathic proteins such as $A\beta$, oligomers may be formed or stabilized in the presence of SDS micelles (Bitan et al., 2005). Therefore, dot blotting experiments were performed on P-2 fractions sonicated in buffer without detergent for 1 of the PD cases and 6 of the AD cases in the series. Due to the low $A\beta$ signal for controls in Western blot analyses and limited cryopreserved tissue volume, dot blot experiments did not include aged normal controls. Fig. 5D demonstrates results generally consistent with Western blotting results, with strong 6E10 and $A\beta$ 42-specific labeling in most AD cases, and strong conformation-dependent A11 immunolabeling of cases showing the strong trimer/tetramer band.

4. Discussion

Soluble oligomeric $A\beta$ species are widely hypothesized to contribute to synaptic failure and early cognitive loss in AD, but the source, sequence of events, and key peptide species in pathogenesis have remained elusive, particularly with respect to human $A\beta$ pathology. Using a series of aged normal subjects, neurologic controls, and AD cases, the present study demonstrates that synaptic terminals contain high levels of soluble oligomers, and $A\beta$ 42 rather than $A\beta$ 40 peptide. $A\beta$ 40 would be the expected dominant species if synaptic $A\beta$ simply reflected production rates. In

size-purified synaptosomes analyzed by flow cytometry, the $A\beta$ signal correlates with the prominent monomer band on Western blots, along with multiple oligomeric $A\beta$ assemblies, C-terminal fragments, and APP.

A role for synaptic $A\beta$ in the progression of AD pathology is suggested by many lines of evidence, including the reduction of hippocampal pathology in APP transgenic mice by lesions of projection axons (Lazarov et al., 2002; Sheng et al., 2002) and the transsynaptic induction of neurofibrillary tangle pathology after $A\beta$ fibril injection (Götz et al., 2001). Activity-dependent synaptic release of $A\beta$ has also been observed (Cirrito et al., 2005), and has been shown to require clathrin-mediated endocytosis (Cirrito et al., 2008). These authors suggest $A\beta$ generation and release at or near the synapse following APP internalization associated with synaptic vesicle recycling. This hypothesis is supported by the present results showing increased APP in AD by Western blot analysis but not in intact synaptosomes, suggesting endosomal APP location. The present results are also directly supported by the recent observation of $A\beta$ release from TgCRND8 synaptoneuroosomes. TgCRND8 mice overexpress 2 APP mutations; in these experiments blockade of release by a glutamate antagonist suggested a potential therapeutic target (Kim et al., 2010).

Dimeric $A\beta$ in synaptosomes has been inversely associated with Mini Mental State Examination (MMSE) (Williams et al., 2009), and recently both monomeric and di-

meric A β from soluble and detergent extracts was strongly associated with dementia in AD cases (Mc Donald et al., 2010). The present observation of abundant monomer but little free dimer in synaptosome-enriched samples, even when probed with multiple antibodies, may result from preparation and/or exposure issues because A β immunoprecipitation was not performed in the present experiments and short exposure times are optimal for quantification. Alternatively, it is possible that dimeric A β in our samples is bound to apoE or other carrier proteins within presynaptic terminals and detected as a larger oligomer on immunoblots. The abundance of synapse-associated A β that runs as monomer on SDS gels is consistent with recent observations of high monomer levels in AD cortex (Mc Donald et al., 2010). As suggested by Mc Donald and colleagues, the high levels of synapse-associated A β monomer and small oligomers observed here may be membrane-associated and represent the active synaptotoxic peptide species. This hypothesis is consistent with extensive evidence that A β oligomers potently inhibit long-term potentiation (LTP) (Selkoe, 2008), control synaptic levels of the NMDA receptor (Snyder et al., 2005), and induce dendritic remodeling (Lacor et al., 2007; Shankar et al., 2008). However, the relatively high signal for A β monomer and soluble oligomers in disrupted synaptosomes and in Western blots relative to intact synaptosomes analyzed by flow cytometry seems more likely to indicate sequestration of A β species within intrasynaptic compartments, consistent with internalization and breakdown but not clearance of SDS-stable A β assemblies within surviving AD terminals. A β monomer may also be generated from higher order oligomers during Western blot analysis procedures. Alternatively, increased A β monomer in surviving terminals may also indicate active synaptic A β generation.

Gamma-secretase activity required for A β production has been localized to endosomes and synaptic membrane and vesicle fractions in rat brain and cell culture (Frykman et al., 2010), and processing of the γ -secretase substrate EphA4 is enhanced by synaptic activity (Inoue et al., 2009). The volume of early endosomes is markedly increased early in AD; early endosomes are close to the cell surface, prominent in processes, and have been suggested as a likely site for upregulated APP processing and A β generation in AD (Cataldo et al., 1997). Taken together with the A β generation observed in studies of released interstitial A β , the observation here of monomers and multiple oligomeric assemblies together with increased APP and AD-associated C-terminal fragments suggests that significant APP processing occurs within a large fraction of cortical synapses, within endosomes, or along the synaptic cleft. However, given that considerable membrane recycling occurs at the synapse following vesicular release, a significant fraction of synaptosome-associated A β peptides might result from internalization of extracellular A β .

A good deal of evidence supports endosomal/lysosomal

structures as a likely location for A β within AD synapses. Ultrastructural analysis in transgenic mouse models and human AD sections has previously shown association of A β with late endosomes near synapses (Takahashi et al., 2002, 2010). Moreover, like other components of the endosomal/lysosomal system, autophagic vacuoles are robustly upregulated in AD cortex (Cataldo et al., 1997; Nixon et al., 2005), which has been suggested to follow from impaired clearance of autophagic vacuoles (Boland et al., 2008). A β monomer has been shown to have neuroprotective functions via antioxidant and insulin-like growth factor-mediated pathways (Giuffrida et al., 2009; Zou et al., 2002). Therefore, in the present study the high level of synaptic A β monomer may have played a role in the survival of A β -bearing terminals in the primarily late stage cases. Indeed, enhancement of lysosomal function has been associated with restoration of synaptic markers in mouse models of AD (Butler et al., 2006), suggesting that a compensatory lysosomal upregulation may slow synapse loss and cell death. Reduced synaptic A β monomer degrading enzymes in AD might account for the elevated A β monomer levels.

Both PD cases in the current series showed multiple synaptic A β oligomers with an A β 42-specific antibody. Consistent with observations that mixed AD/PD pathology progresses more rapidly, the PD case with a 2-year history of dementia closely resembled pathology in the AD cases. A diagnosis of Lewy body variant AD was considered for this case because of the mild to moderate plaque pathology, but this case did not display neurofibrillary tangles, and the Lewy bodies were primarily in the brainstem rather than throughout the cortex. Neither dementia nor plaque/tangle pathology was observed in the other PD case, which showed prominent low molecular weight oligomers labeled by the conformation-dependent antibody A11. The synaptic A β pathology we observe in PD cases is in line with observations that A β and α -synuclein coimmunoprecipitate in patients with AD/PD and in transgenic mice (Tsigelny et al., 2008), and with a hypothesis that interactions between misfolded proteins may contribute to augmentation of pathology in mixed neurodegenerative disease.

The polyclonal antibodies A11 and OC were raised against prefibrillar and fibrillar oligomers, respectively. However, the present results also demonstrate significant sensitivity to A β tertiary structure with monoclonal antibodies, because differential labeling of A β assemblies was observed in denatured samples in Western blot analysis. Conformational sensitivity is confounded to some degree with sequestration within intrasynaptosomal compartments such as lysosomes, as illustrated by differential labeling in situ (flow cytometry of intact synaptosomes), compared with dot blots of native proteins from mechanically disrupted synaptosomes. The previously unrecognized conformational sensitivity of monoclonal antibodies, along with the abundance of synaptic A β in our studies likely results from our extensive use of unfixed, short post mortem inter-

val (PMI) tissue that is cryopreserved. This conclusion is in line with the observation that epitope masking has historically limited A β detection in situ, and is a factor contributing to false-negative results for intraneuronal localization of A β (Gouras et al., 2005).

In addition to serving as a possible seed for extracellular deposits, high levels of synaptic A β may be an upstream initiator of protein misfolding and neurodegeneration-related cascades, including p-tau pathology and disruption of axonal transport and synaptic transmission. These cascades are likely to be complex and interrelated, and signal a need for continued exploration with the ultimate goal of preventing early stages of synaptic dysfunction and cognitive decline.

Disclosure statement

There are no conflicts of interest to report for any of the authors.

Appropriate approvals and procedures were followed for the use of human postmortem tissue.

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