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Diffusible, highly bioactive oligomers represent a critical minority of soluble A β in Alzheimer's disease brain

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Abstract

Significant data suggest that soluble A β oligomers play an important role in Alzheimer's disease (AD), but there is great confusion over what exactly constitutes an A β oligomer and which oligomers are toxic. Most studies have utilized synthetic A β peptides, but the relevance of these test tube experiments to the conditions that prevail in AD are uncertain. A few groups have studied A β extracted from human brain, but they employed vigorous tissue homogenization which is likely to release insoluble A β that was sequestered in plaques during life. Several studies have found such extracts to possess disease-relevant activity and considerable efforts are being made to purify and better understand the forms of A β therein. Here, we compared the abundance of A β in AD extracts prepared by traditional homogenization versus using a far gentler extraction, and assessed their bioactivity via real-time imaging of iPSC-derived human neurons plus the sensitive functional assay of long-term potentiation. Surprisingly, the amount of A β retrieved by gentle extraction constituted only a small portion of that released by traditional homogenization, but this readily diffusible fraction retained all of the A β -dependent neurotoxic activity. Thus, the bulk of A β extractable from AD brain was innocuous, and only the small portion that was aqueously diffusible caused toxicity. This unexpected finding predicts that generic anti-oligomer therapies, including A β antibodies now in trials, may be bound up by the large pool of inactive oligomers,

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Author Contributions

DMW conceived the idea, directed the research, analyzed data and wrote the manuscript. WH conducted biochemical and IncuCyte experiments, analyzed data, prepared figures and assisted with writing the manuscript. ZW did all electrophysiological recordings, analyzed data and prepared figures. WL cultured iPSC-derived neurons and assisted with biochemical experiments. TTO'M trained WH and conducted initial biochemical experiments. MJ provided critical guidance for the use of iPSC-derived neurons and the IncuCyte. MW and CH provided 2E9 antibody, recombinant APP₅₀₅₋₆₁₂ and furnished critical guidance on the detection of APP fragments. MF performed postmortem analyses and assessed the disease status of patient brains. All authors critically reviewed the manuscript.

whereas agents that specifically target the small pool of diffusible, bioactive A β would be more useful. Furthermore, our results indicate that efforts to purify and target toxic A β must employ assays of disease-relevant activity. The approaches described here should enable these efforts, and may assist the study of other disease-associated aggregation-prone proteins.

Keywords

Amyloid β -protein; automated live-cell imaging; iPSC-derived human neurons; long-term potentiation; neuritic dystrophy; soluble aggregates

Introduction

Based on our current understanding of Alzheimer's disease (AD) genetics and longitudinal biomarkers, it seems probable that the amyloid β -protein (A β) plays an initiating role in this disease [14, 40]. A β can exist in multiple different forms, but the relative pathogenic importance of these different forms is unknown. To date, most efforts to investigate A β structure and/or bioactivity have utilized synthetic peptides [61]. Although rightly criticized for their potential lack of relevance to the human disease, these studies have helped define certain basic parameters. Specifically, the majority of such reports indicate that A β monomers are not directly neurotoxic, but have to aggregate to become toxic [61] and that the process of aggregation is required for toxicity [2, 52]. While some studies indicate that the active growth of A β aggregates imparts toxicity [15, 58], most investigators have focused on the generation of toxic oligomeric intermediates [2, 50]. However, oligomers are by nature, dynamic, difficult to isolate, and thus tricky to study. As a result, it is not clear which, if any, of the long list of synthetic A β oligomer species are relevant to AD [2, 43].

Given, the widespread interest in A β oligomers, it is surprising that limited efforts have been made to characterize and study soluble forms of A β isolated from human brain [4, 55]. Relevant studies define soluble A β operationally as any form of the peptide that remains in aqueous solution following high speed centrifugation [23, 55]. Typically, cortical tissue is homogenized in aqueous-buffer and then centrifuged at high speed and the supernatant removed and analyzed. Regrettably, the methods used to prepare homogenates, the ratio of tissue to buffer, the composition of buffer and the centrifugation conditions applied, vary widely both between groups and in publications from the same groups. Nonetheless, certain common themes have emerged and it is clear that A β extracted from AD brain in aqueous buffer comprises a mixture of different-sized assemblies [23, 25, 39, 42, 49] and that one or more of these components are potent neurotoxins [1, 3, 5, 9, 17, 34, 42, 48, 54]. However, the molecular space between innocuous A β monomers and end-stage amyloid plaques is potentially massive and it seems unlikely that all of the A β assemblies which occupy this large middle ground are toxic. Moreover, it is unclear whether the clarified brain extracts studied (by us and the field in general) accurately represent the truly diffusible A β expected to be present in the interstitial fluid of AD brain.

In an effort to address these important issues, we compared the A β content and neurotoxic activity of AD brain extracts prepared by traditional crude homogenization versus more gentle extraction. Bioactivity was assessed using two distinct AD-relevant readouts: real-

time imaging of iPSC-derived human neurons, and measurement of long-term potentiation in mouse hippocampus. Matched pieces of gray matter from the same AD or control brains were either: (i) Dounce homogenized (the traditional approach), or (ii) soaked in buffer to capture diffusible species, and the suspensions then clarified by high speed centrifugation (Figure 1). Both the homogenized (**H**) and buffer-soaked (**S**) AD brain extracts disrupted neurites and blocked LTP to a comparable extent, and in each case toxicity was prevented by specific removal of A β . However, when we measured A β , we were surprised to find that our **S extracts** contained much lower amounts of oligomers than did the traditional **H extracts**. Moreover, when **H extracts** were diluted to match the A β content measured in corresponding **S extracts** from the same tissue block, the diluted **H extracts** had greatly reduced or no activity. Similarly, homogenization of the tissue pellet remaining after **S** extraction yielded a fraction rich in A β , but lacking in toxic activity. Collectively, these results indicate that intrinsic diffusibility is a key requirement for, and predictor of, toxicity, and that the bulk of soluble brain A β is innocuous, and only a small portion is toxic.

Several important learnings flow from these unexpected findings. *First*, therapeutic agents targeting A β oligomers would be most effective if they were specific for the small pool of toxic A β , and avoided the much more abundant non-toxic A β oligomers. *Second*, current efforts to purify toxic oligomers [7] are flawed because they use brain extracts produced by crude mechanical disruption, and so the majority of A β present is inactive. *Third*, our paradigm sets the stage to examine the relationship between toxicity and seeding, and offers an experimental approach toward understanding the lack of correlation between amyloid burden and cognitive impairment. *Finally*, while here we focused solely on A β , it seems likely that the approaches developed in this study will be applicable to diffusible oligomers formed by other disease-associated proteins.

Materials and Methods

Reagents and chemicals

A β 1–40 and A β 1–42 peptides were synthesized and purified using reverse-phase HPLC by Dr. James I. Elliott at the ERI Amyloid laboratory, Oxford, CT, USA. Peptide mass and purity (>99%) were confirmed by reverse-phase HPLC and electrospray/ion trap mass spectrometry. N-terminally extended (NTE) –31A β –40 was prepared and purified as described previously [47] and recombinant A η - α (APP₅₀₅₋₆₁₂) was a gift from Drs. M. Willem and C. Haass (Ludwig-Maximilian University, Munich). A η - α peptide was dissolved in 50 mM ammonium bicarbonate, pH 8.5, diluted to 10 ng/ μ l, aliquoted, and stored frozen at –80°C. A β and NTE-A β were dissolved in 50 mM Tris-HCl, pH 8.5, containing 7 M guanidium-HCl (GuHCl) and 5 mM ethylenediaminetetraacetic acid (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 minutes at 16,000 g and chromatographed on a Superdex 75 10/300 GL column eluted at 0.5 ml/min with 50 mM ammonium bicarbonate, pH 8.5. The concentration of the peak fraction for each peptide was determined from its absorbance at 275 nm. Peptide was then diluted to 10 ng/ μ l with the same buffer used for SEC, aliquoted and stored frozen at –80°C. When needed, an aliquot of a given peptide was thawed, used, and any remaining sample was discarded. Gel filtration

standards were purchased from Bio-Rad (Hercules, CA). All other chemicals were of the highest purity available and unless indicated otherwise were obtained from Sigma-Aldrich (St. Louis, MO). For experiments involving A β peptides or brain extracts, protein Lo-Bind tubes (Eppendorf, Hamburg, Germany) were used.

Antibodies

The antibodies used in this study and their sources are described in Table 1.

Preparation of human brain extracts

Human specimens were obtained from the Massachusetts ADRC Neuropathology Core, Massachusetts General Hospital and used in accordance with the Partners Institutional Review Board (Protocol: Walsh BWH 2011). Frozen temporal cortical tissues were obtained from a total of 10 cases, 9 of whom died with end-stage AD, and 1 subject who died free of AD (Table 2). All AD cases met current post-mortem and clinical diagnostic criteria. Post-mortem intervals were less than 48 hours.

Approximately 20 g of cortical gray matter was dissected from each case and this material was then sliced into ~2 g lots with a razor blade. Each lot was further cut into small chunks using a McIlwain tissue chopper (set at 0.5 mm). The diced tissue was gently mixed and divided in two. One half was used to prepare **H extract** and the other to prepare **S extract** (Fig. 1). Both extracts were prepared using a buffer that we refer to as artificial cerebrospinal fluid base buffer (aCSF-B) (124 mM NaCl, 2.8 mM KCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, pH 7.4). aCSF-B is the core buffer used in subsequent electrophysiology experiments. For preparation of brain extracts aCSF-B was supplemented with protease inhibitors (5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethyleneglycoltetraacetic acid, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin, 2 μ g/ml pepstatin, 120 μ g/ml pefabloc and 5 mM NaF).

H extracts were prepared by homogenizing tissue in 5 volumes of ice-cold aCSF-B with 25 strokes of a Teflon-glass Dounce homogenizer (Fisher, Ottawa, Canada). Resulting 20% (w/v) homogenates were centrifuged at 200,000 g for 110 minutes and 4°C in a SW41 Ti rotor (Beckman Coulter, Fullerton, CA). The upper 80% of the supernatant was removed and designated as **H extract**. **S extracts** were prepared by incubating tissue in 5 volumes of ice-cold aCSF-B at 4°C for 30 minutes with gentle side-to-side mixing. Thereafter, this suspension was centrifuged at 2,000 g for 10 minutes and 4°C. The upper 90% of the supernatant was removed, centrifuged at 200,000 g for 110 minutes and 4°C in a SW41 Ti rotor. The resulting supernatant was removed and designated as **S extract**. **S extracts** necessarily include molecules derived from extracellular and intracellular compartments because prior to extraction, tissue underwent procedures that cause the rupture of cells (e.g. autolysis during the postmortem interval, freezing and thawing, dissecting, and slicing tissue). **H2 extracts** were prepared using the pellets generated when preparing **S extracts**. The 2,000 g and 200,000 g pellets were pooled and homogenized in 5 volumes of ice-cold aCSF-B, and centrifuged at 200,000 g for 110 minutes and 4°C. The upper 80% of supernatant was removed and designated as **H2 extract**.

H, *S* and *H2 extracts* were then dialyzed against fresh aCSF-B. Fifty ml of extract was dialyzed (using Slide-A-Lyzer™ G2 Dialysis Cassettes, 2K MWCO, Fisher Scientific) against a 100-fold excess of fresh aCSF-B at 4°C, with buffer changed 3 times over a 72 hour period. Dialysis was used to remove small molecules such as excitatory amino acids and drugs that might interfere with our bioactivity assays, and the success of the process was confirmed by measuring the amount of glutamate in the final dialysate versus the starting extract. Thereafter, *extracts* were divided into 2 parts: 1 portion was immunodepleted (ID) of Aβ by 3 rounds of 12 hour incubations at 4°C with the anti-Aβ antibody, AW7, conjugated to Protein A Sepharose (PAS) beads [44]. The second portion was treated in an identical manner, but this time incubated with pre-immune serum conjugated to PAS beads. Samples were cleared of beads and 0.5 ml aliquots stored at -80°C until used for biochemical or bioactivity experiments. Samples were thawed once and used.

Measurement of soluble proteins in brain extracts

Total protein content in *H*, *S* and *H2 extracts* was measured using a Pierce BCA assay kit (ThermoFisher, Waltham, MA) in accord with suppliers' instructions. Briefly, samples were diluted to 1:5 with aCSF-B and analyzed in triplicate versus bovine serum albumin (BSA) standards also prepared in aCSF-B with serial dilutions of BSA ranging from 0-2 mg/ml. To detect sAPP and BDNF, *H*, *S* and *H2 extracts* were mixed with equal volumes of 2× sample buffer and 10 µl of this was loaded in a single well and electrophoresed on either a pre-cast 16% polyacrylamide tris-tricine gel (for detection of BDNF) or a 10% polyacrylamide tris-glycine gel (for detection of sAPP) (Invitrogen, Carlsbad, CA). Gels were rinsed in transfer buffer (10% methanol, 0.192 M glycine, and 25 mM Tris) and transferred onto 0.2 µm nitrocellulose at 400 mA and 4°C for 2 hours. Membranes were blocked with 50% Odyssey blocking buffer in PBS for 1 hour at RT and then probed with appropriate antibodies. Monoclonal antibody 22C11 (Millipore, Billerica, MA) was used to detect sAPP and rabbit polyclonal antibody N-20 (Santa Cruz, Dallas, TX) was used to detect BDNF. Bands were visualized using a Li-COR Odyssey infrared imaging system (Li-COR, Lincoln, NE). The relative intensity of protein bands was determined and these values were used to estimate the percentage of sAPP and BDNF in *S* or *H2* relative to *H*, i.e. S/H or $H2/H \times 100$.

Monomer-preferring MSD Aβ immunoassays

The Aβx-40 and Aβx-42 assays preferentially detect Aβ monomers ending at Val40 and Ile 42, respectively. The x-40 assay uses monoclonal antibody (mAb) m266 (3 µg/ml), for capture and biotinylated 2G3 (0.2 µg/ml) for detection; the x-42 assay uses m266 (3 µg/ml) for capture and biotinylated 21F12 (0.4 µg/ml) for detection. Since incubation of samples with GuHCl dissociates soluble Aβ aggregates allowing increased detection of monomer by the Aβx-40 and Aβx-42 assays [29], samples were analyzed with and without pre-incubation in 5 M GuHCl. Briefly, 20 µl of extract was incubated with 50 µl of 7 M GuHCl at 4°C overnight. Thereafter samples were diluted 1:10 with assay diluent so that the final GuHCl concentration was 0.5 M. To match the buffer composition of standards with samples, monomeric stocks of Aβ1-40 and Aβ1-42 were prepared in Tris-buffered saline, pH 7.4 containing 0.5 M GuHCl, 0.05% Tween 20 and 1% Blocker A. Assays were performed using the Meso Scale Discovery (MSD) platform and reagents from Meso Scale (Rockville,

MD). Samples, standards and blanks were loaded in triplicate and analyzed as described previously [28].

Oligomer-preferring MSD A β immunoassay

This oAssay is >37,000-fold more selective for A β oligomers/soluble aggregates than A β monomer and uses amyloid-derived diffusible ligands as the calibrant [60]. The assay is performed essentially as described for the monomer-preferring assays, but employs the aggregate-preferring mAb, 1C22, for capture (3 μ g/ml) and biotinylated 3D6 (0.4 μ g/ml) for detection [28, 60]. When A β aggregates are treated with GuHCl, the signal of this assays is greatly attenuated [29]. The percentage of different forms of A β in *S* or *H2* relative to *H* was estimated using the values from the above 5 assays.

Culture of Chinese hamster ovary (CHO) cell lines

Media, fetal bovine serum (FBS), and media supplements were from Invitrogen (Carlsbad, CA). Naive, untransfected CHO cells were grown in Dulbecco's modified Eagles medium (DMEM) containing 10% FBS, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine. CHO cells stably transfected with human APP751 bearing the V717F mutation (which we refer to as 7PA2 cells) were grown in CHO medium plus G418 (200 μ g/ml) [36]. Once cells reached 95-100% confluency, they were washed with 5 ml serum-free medium and incubated in 5 ml serum-free medium for an additional ~15 hours. Thereafter, medium was removed and centrifuged at 4°C and 200 g for 10 min. The upper 90% of the supernatant was transferred to a clean tube and centrifuged at 4°C and 3,000 g for a further 10 minutes. The upper 90% of the supernatant was removed and 5 mM EDTA was added to inhibit proteolysis. Finally, media was aliquoted into 2 ml lots and stored at -80°C.

Immunoprecipitation/Western blot analysis of amyloid β -protein

Extracts were first pre-cleared with PAS beads to minimize non-specific interactions in the subsequent IP. One ml aliquots of extracts were incubated with 15 μ l PAS beads for 1 hour at 4°C with gentle shaking. PAS beads were removed by centrifugation (4,000 g for 5 minutes) and the supernatant divided into 0.5 ml aliquots. Each aliquot was incubated with 10 μ l of AW7 and 15 μ l PAS beads overnight at 4°C with gentle shaking. A β -antibody-PAS complexes were collected by centrifugation and washed as previously described [54]. The immunoprecipitated (IP'd) A β was eluted by boiling in 15 μ l of 2 $\times$ sample buffer (50 mM Tris, 2% w/v SDS, 12% v/v glycerol with 0.01% phenol red) and electrophoresed on hand poured, 15 well 16% polyacrylamide tris-tricine gels. Synthetic A β 1-42 was run as a loading control and protein transferred onto 0.2 μ m nitrocellulose at 400 mA and 4°C for 2 hours. Blots were microwaved in PBS and A β detected using the anti-A β 40 and anti-A β 42 antibodies, 2G3 and 21F12, and bands visualized using a Li-COR Odyssey infrared imaging system (Li-COR, Lincoln, NE). For certain experiments, the relative intensity of the ~4 kDa and ~7 kDa A β bands was determined and these values were used to estimate the percentage of species in *S* or *H2* relative to *H*, i.e. *S/H* or *H2/H* $\times$ 100.

To determine if AW7 IP'd non-A β APP metabolites (e.g. sAPP, N-terminally extended A β or A η peptides) from AD brain extracts, one milliliter aliquots of 7PA2 condition medium (7PA2-CM) or half milliliter aliquots of AD4 *H extract* were IP'd with either AW7

antisera, or pre-immune serum (PI). The supernatant of AW7 IP'd 7PA2-CM was buffer-exchanged into 50 mM ammonium bicarbonate, pH 8.5, using a Zeba spin desalting column, lyophilized, and used for SDS-PAGE. Western blots were developed with 2E9, 6E10, or HJ2 plus 21F12 (Table 1) and detected using ECL+ (Thermo Fisher Scientific, Rockford, IL).

Size exclusion chromatography

Samples were chromatographed on a Superdex 200 10/300 GL column eluted with 50 mM ammonium bicarbonate, pH 8.5 at 0.5 ml/minute. The column outlet was attached directly to a fraction collector and the elution of standards was monitored off line using a spectrophotometer. Each day prior to analyzing samples, the column was calibrated using Blue dextran and gel filtration standards. The peak fraction containing Blue dextran was designated as fraction zero. Two 0.5 ml aliquots of *H* or *S* extracts were removed from -80°C , thawed at room temperature for 20 minutes, pooled, vortexed and centrifuged at 12,000 rpm for 10 minutes. The upper 0.95 ml of sample was removed and loaded onto the SEC column and 0.6 ml fractions collected. To enable detection of A β of different aggregation states, fractions were lyophilized, then reconstituted in 60 μl of 5 M GuHCl and incubated at 4°C overnight. Thereafter, samples were diluted 1:10 with assay diluent and analyzed using the MSD-based A β x-42 assay. To avoid cross-contamination of samples, no more than 3 brain samples were chromatographed on any given day, and in between samples 1 ml of 5 M GuHCl was loaded onto the column and eluted with at least 2 column volumes of buffer. At the end of each day, the column and collection tubing were thoroughly washed as described previously [44].

Experiments to isolate monomeric A β or NTE-A β peptides were performed using a Superdex 75 10/300 GL column connect to a BioRad BioLogic DuoFlow Chromatography System and eluted with 50 mM ammonium bicarbonate, pH 8.5 at 0.5 ml/minute.

Production of induced neurons (iNs) from human induced pluripotent stem cells (iPSCs)

Neurogenin 2 (Ngn2)-induced human neurons [63] were prepared as summarized in Supplementary Fig. 1 and as described previously [16]. Briefly, YZ1 iPSCs [62] were maintained in media containing DMEM/F12, Knockout Serum Replacement, penicillin/streptomycin/glutamine, MEM-NEAA, and 2-mercaptoethanol (all from Invitrogen, Carlsbad, CA) plus 10 $\mu\text{g/ml}$ bFGF (Millipore, Billerica, MA). iPSCs were plated at a density of 95,000 cells/ cm^2 for viral infection. Lentiviruses were obtained from Alstem with “ultrapure titres” and used at the following concentrations: pTet-O-NGN2-puro: 0.1 $\mu\text{l}/50,000$ cells; Tet-O-FUW-eGFP: 0.05 $\mu\text{l}/50,000$ cells; Fudelta GW-rtTA: 0.11 $\mu\text{l}/50,000$ cells. To induce Neurogenin 2 expression doxycycline was added on “iN day 1” (Supplementary Fig. 1) at a concentration of 2 $\mu\text{g/ml}$. On iN day 2, puromycin was added at 10 mg/ml and maintained in the media at all time thereafter. On iN day 4, cells were plated at 5,000 cells/well on Matrigel (Corning, NY) coated Greiner 96 well microclear plates and maintained in media consisting of Neurobasal medium (Gibco), Glutamax, 20% Dextrose, MEM-NEAA and B27 with BDNF, CNTF, GDNF (PeprTech, Rocky Hill, NJ) each at a concentration of 10 ng/ml . Prior studies indicated that neurite number and expression of neural markers reached near maximal levels by iN day 14 and that iNs were fully mature by

iN day 21 [16]. To investigate the effects of AD brain extracts on neuritic integrity, cells were used at iN day 21.

Addition of AD brain extract to induced neurons (iNs) and live-cell imaging

Brain extracts were thawed on ice for 30-60 minutes, vortexed, centrifuged at 16,000 g for 2 minutes, and exchanged into neurobasal medium supplemented with B27/Glutamax using a HiTrap 5 ml desalting column (GE Healthcare, Milwaukee, WI). Briefly, two, 0.5 ml aliquots were pooled and applied to a desalting column using a 1 ml syringe at a flow rate of ~1 ml/min and eluted with iN culture medium. Ten, 0.5 ml fractions were collected. Prior studies indicated that fractions 4 and 5 contained the majority of eluted A β . Consequently, fractions 4 and 5 were pooled and used in subsequent iN experiments. A small portion (50 μ l) of this material was also taken for A β analysis.

Approximately 7 hours prior to exchanging AD brain extracts into culture medium, iN day 21 neurons (Supplementary Fig. 1) were placed in an IncuCyte Zoom live-cell imaging instrument (Essen Bioscience, Ann Arbor, MI) and images collected every 2 hours for a total of 6 hours. This analysis was used to define neurite length prior to addition of brain extracts. Immediately after the acquisition of baseline images, half of the medium on iNs was removed (leaving ~100 μ l) and 50 μ l of buffer-exchanged extract or vehicle, plus 50 μ l of fresh medium was added.

Thereafter, images were collected from four fields per well every 2 hours for a total of 84 hours. Phase contrast images sets were analyzed using Incucyte Zoom 2016A Software (Essen Bioscience, Ann Arbor, MI). The 'NeuroTrack' analysis job was used to automatically define neurite processes and cell bodies [16]. Typical settings were: Segmentation Mode = Brightness; Segmentation Adjustment = 1.2; Cell body cluster filter = minimum 500 μ m²; Neurite Filtering = Best; Neurite sensitivity = 0.4; Neurite Width = 2 μ m. Total neurite length (in mm) was quantified and normalized to the average value measured during the 6 hour period prior to sample addition.

Immunocytochemical analysis of induced neurons (iNs) and confocal microscopy

At the end of certain experiments, iNs were fixed, stained and used for confocal microscopy. Cells were fixed in 4% paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, PA) and 4% sucrose at room temperature for 15 minutes, and then permeabilized with ice-cold methanol for 3 minutes. Cells were washed 3 times with PBS and then blocked using 5% (w/v) BSA in PBS containing 0.3% Triton X-100 and 0.02% sodium azide. Thereafter, iNs were incubated overnight with primary antibody (mouse anti- β -tubulin, Millipore, Billerica, MA; 2 μ g/ml) at 4°C. Cells were again washed with PBS (x3) and then incubated for 1 hour at room temperature with fluorescence-conjugated secondary antibodies (AlexaFluor 546 goat anti-mouse; Invitrogen; at 2 μ g/ml). Finally, iNs were incubated with DAPI (1 μ g/ml in PBS, Invitrogen) for 15 minutes, washed 3 times with PBS and examined using a Zeiss LSM710 confocal microscope fitted with a 40x air objective (NA: 0.8). Images were captured in a Z-stack manner (15 stacks, interval 2 μ m) and maximal pixel intensity projections were created with averaging of 2 frames set to 1024 $\times$ 1024 pixel resolution.

Mice

All animal procedures were performed in accordance with the National Institutes of Health Policy on the Use of Animals in Research and were approved by the Harvard Medical School Standing Committee on Animals. Wild type (WT) C57BL/6 mice were purchased from Jackson Labs (Bar Harbor, ME) and a small colony maintained in-house. Animals were housed in a room with a 12 hour light/dark circadian cycle with *ad libitum* access to food and water.

Brain slice preparation

Both male and female animals were used. At 2-3 months of age, mice were anaesthetized with isoflurane and decapitated. Brains were rapidly removed and immediately immersed in ice-cold (0-4°C) artificial cerebrospinal fluid (aCSF). The aCSF contained (in mM): 124 NaCl, 3 KCl, 2.4 CaCl₂, 2 MgSO₄·7H₂O, 1.25 NaH₂PO₄, 26 NaHCO₃ and 10 D-glucose, and was equilibrated with 95% O₂ and 5% CO₂, pH 7.4, 310 mOsm. Coronal brain slices (350 µm) including hippocampus [54] were prepared using a Leica VT1000 S vibratome (Leica Biosystems Inc, Buffalo Grove, IL) and transferred to an interface chamber and incubated at 34 ± 5°C for 20 minutes and then kept at room temperature for 1 hour before recording.

Long-term potentiation (LTP) recording

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

Application of samples to LTP bath media

Samples were stored frozen at -80°C in 0.5 ml aliquots and allowed to thaw at room temperature for 10 minutes and gently mixed by hand before using. For experiments when *H extracts* were diluted, stocks were thawed and diluted with aCSF-B immediately prior to use

for LTP experiments. After a stable baseline had been achieved for at least 10 minutes, samples were added to the aCSF reservoir. The total volume in the reservoir, the recording chamber, the tubing and the pump was 9.5 ml, such that the effective dilution of each sample was 1:20. Thirty minutes after addition of sample, a TBS was delivered to induce LTP as described above. The experimenter was blinded to the identity of the *H*, *S*, ID, D, and aCSF samples, and samples were tested in an interleaved manner to avoid variances in animals or slice quality. Slices in each group came from different animals unless otherwise noted.

Statistical analysis

Electrophysiological data were analyzed offline by pCLAMP 10.2 (Molecular Devices, Sunnyvale, CA) and tested with One-way analysis of variance (ANOVA) with Bonferroni *post-hoc* tests or student *t*-tests. For live-cell imaging experiments, differences between groups were tested with ANOVA with Bonferroni *post-hoc* tests or student *t*-tests.

Results

We and others have shown that clarified crude homogenates of AD brain exert a range of activities relevant to AD and that these effects are reversed by certain anti-A β antibodies [1, 3, 9, 16, 17, 42, 54]. Whether the bioactive agents in these extracts are truly extracellular, soluble and diffusible in brain, or are artifacts of the extraction process is uncertain. Here we describe a gentle extraction procedure that allows the release of soluble A β with disease-relevant activity.

The majority of soluble proteins can be recovered from cortical gray matter by soaking tissue in aqueous buffer

An initial goal was to develop an extraction procedure that releases the maximum amount of aqueous soluble proteins from brain tissue while minimizing disruption of that tissue. Since truly soluble extracellular proteins should be able to diffuse within tissue and into aqueous solutions surrounding tissue we reasoned that cutting gray matter into thin pieces and then soaking these pieces in buffer would allow the transfer of soluble proteins from brain tissue into buffer. To directly compare recovery of proteins produced using our new procedure versus traditional homogenization, we used a McIlwain tissue chopper to dice 2 g pieces of cortical gray matter into small chunks. This material was then gently mixed and divided in two (Fig. 1). One portion was homogenized in aCSF-B using a Teflon-glass Dounce homogenizer and then clarified by high-speed centrifugation. Since this extract involves a homogenization step it is referred to as *H extract*. The other portion was incubated[†] in aCSF-B with gentle side-to-side mixing. After incubation, the intact tissue was removed by low speed centrifugation. High-speed centrifugation was avoided at this stage since the force of tissue hitting the bottom of the tube could potentially release otherwise insoluble material. After low speed centrifugation, the supernatant was removed to a new tube and spun at high speed to remove microvesicles and particulates. Given that the production of such extracts involves a step in which brain tissue is “soaked” in buffer, it is referred to as *S extract*. Since

[†]In preliminary experiments (Supplementary Fig. 2) we investigated the time required to allow efficient release of protein into *S extract*. Protein release reached a plateau between 15-30 minutes. Thus, we set our incubation period to 30 minutes.

H extracts should contain the same material as in **S extracts** plus material released by homogenization, we re-extracted the **S extract** pellets by homogenizing them in aCSF-B. Because this material is produced by homogenization it is denoted as **H2 extract**. **H**, **S** and **H2 extracts** were prepared from 9 end-stage AD cases and 1 control (Table 2). Thereafter, we measured the levels of total protein and two specific extracellular brain proteins in each of the extracts.

The amount of detectable protein in the 10 **S extracts** ranged from 2.2 mg/ml to 3.9 mg/ml and in the **H extract** from 2.8 to 3.8 mg/ml (Fig. 2a). In general, there tended to be more protein in **H extracts** than in **S extracts**, and on average **S extracts** contained 88% as much protein as **H extracts**. For extracts from the same brain the relative amount of protein in **S extracts** ranged from 73% to 110% (Fig. 2d and Table 3). The relative levels of sAPP followed a similar pattern (Fig. 2b and Supplementary Fig. 3a). The percentage of sAPP in **S extracts** relative to **H extracts** ranged from 64% to 104%, and as with total protein, the average level of sAPP in **S extracts** was 88% (Fig. 2d and Table 3). sAPP is a medium sized extracellular protein with molecular weight >70 kDa [35, 45]. Thus, it would appear that the majority of such proteins can readily diffuse out of gray matter when tissue is incubated in aqueous buffer. Secreted BDNF is a relative small protein of ~14 kDa [19] and as such one would anticipate that BDNF would more readily diffuse out of brain tissue than sAPP. Indeed, we found that the **S extract** from all 10 brains contained at least 86% as much BDNF as **H extract**, and on average 97% of BDNF detected in **H extracts** was recovered in the **S extract** (Fig. 2c and d, Supplementary Fig. 3b and Table 3). These results indicate that freely soluble A β monomer should readily diffuse into buffer in which brain tissue is soaked. The sAPP results also suggest that A β oligomers composed of up to ~15 monomers should be readily recovered in the **S extract**. Importantly, the levels of total protein, sAPP and BDNF were much lower in **H2 extracts** (Fig. 2 and Table 4). For instance, on average **H2 extracts** contained only 22% of the BDNF detected in **H extracts**. These results indicate that **H2 extracts** contain only very modest levels of truly diffusible molecules, with the relative content of soluble protein conforming to the equation: $H \sim S + H2$, whereas $S > H2$.

Homogenization allows release of substantially more A β than soaking tissue in aqueous buffer

Having determined that soaking tissue in aCSF-B allows excellent recovery of soluble proteins, we went on to examine the forms and relative amounts of A β present in **S** and **H extracts**. A β is highly heterogeneous in terms of primary structure and aggregation state [52], hence we employed a series of detection and separation methods. First, we focused on the measurement of A β using highly sensitive immunoassays. This included the use of 2 monomer-preferring immunoassays, one for A β 40 and the other for A β 42, and an immunoassay that preferentially detects soluble A β aggregates. No A β was detected in the **H**, **S** and **H2 extracts** from the one control case examined (Supplementary Tables 1–3). For the 9 AD brains, the amount of A β x-40 monomer in **S extracts** relative to **H extracts** varied widely from case to case, but in 5 out of 9 cases relatively high amounts of A β 40 monomer were recovered in **S extracts** (Fig. 3a and Supplementary Table 1). The absolute concentration of A β 42 monomer in both **S** and **H extracts** tended to be slightly higher than A β 40 monomer and the amount of A β 42 was more similar across brains (Fig. 3c and

Supplementary Table 2). On average the amount of A β 42 monomer detected in *S extracts* was 36% of that detected in *H extracts* (Fig. 3f and Table 3). The results of the A β 40 and A β 42 monomer assays indicate that more monomer is detected in extracts prepared by homogenization, thus suggesting that in most cases a large portion of the monomer detected in brain homogenates does not occur in a natively diffusible form, but rather is released by mechanical disruption. This conclusion is supported by the fact that *H2 extracts* always contain the highest levels of A β 40 and A β 42 monomers (Fig. 3a and c, Supplementary Tables 1 and 2). Native measurement of soluble aggregates using our oAssay followed a similar pattern to that seen for native monomers (Fig. 3e). That is, the amount of native aggregates in *S extracts* was similar across brains, but in general was much lower than in *H extracts* (Fig. 3e and Supplementary Table 3). The levels of native aggregates were much higher in *H2 extracts* than in *S extracts*, and were comparable to the levels measured in *H extracts* (Fig. 3e, Table 4 and Supplementary Table 3). Thus, it would appear that the majority of soluble A β aggregates detected in AD brain homogenates requires mechanical disruption of tissue and may arise due to release of this material from diffuse A β deposits, or other normally insoluble sources.

Previously we have shown that soluble A β aggregates found in AD brain homogenates are susceptible to treatment with denaturants such as GuHCl, and that incubation of aggregate-containing extracts with GuHCl allows their disassembly and quantitation using monomer-preferring immunoassays [29]. Here, we measured the levels of A β 40 and A β 42 in extracts after incubation with GuHCl. The levels of A β 40 in the *H*, *S* and *H2 extracts* were only modestly increased after treatment with GuHCl (Fig. 3b and Supplementary Table 1). In contrast, the levels of A β 42 in *H extracts* following GuHCl treatment were on average ~9.1-fold higher, and in *S extracts* there was ~4.4-fold more A β 42 detected after GuHCl treatment (Fig. 3d and Supplementary Table 2). On average *S extracts* contained only a fifth the amount of A β 42 as *H extracts* (Fig. 3f and Table 3). As with the oAssay, the levels of A β 40 and A β 42 in following pre-treatment with GuHCl were much higher in *H2 extracts* than in *S extracts* and were comparable to, or higher than those measured in *H extracts* (Fig. 3f and Table 4). Collectively our immunoassay results indicate that *S extracts* contain only a fraction of the soluble A β aggregates found in AD brain homogenates and that most of the A β found in *H extracts* is released by mechanical disruption, i.e., $H \sim S + H2$, where $H2 > S$.

Diffusible A β migrates at ~4 kDa and ~7 kDa on SDS-PAGE based Western blotting

IP/WB is perhaps the most common method that has been used to detect and measure A β in brain extracts and allows detection of 2 distinct forms of A β , material which migrates on SDS-PAGE with molecular weights consistent with A β monomer (~4 kDa) and a broad band centered around 7 kDa [9, 16, 29, 30, 42, 54]. For IP we used AW7, an anti-A β antibody capable of detecting multiple A β sequences and conformations [29, 31], but which shows no measurable reactivity with non-A β APP metabolites (Supplementary Fig. 4). Analysis of *H extracts* produced patterns and levels of A β comparable with hundreds of brain homogenates we have analysed by IP/WB [29–31, 42]. AD *H extracts*, but not the control extract, contained 2 prominent broad bands: one centered ~4 kDa and the other ~7 kDa (Fig. 4a–c and Supplementary Fig. 5). AD *S extracts* also contained the ~4 and ~7 kDa bands, but

at much lower levels than in *H extracts* (Fig. 4a–c and Supplementary Fig. 5a–d). In 7 out of 9 cases *S extracts* contained less than 5% of the ~4 kDa A β species detected in the corresponding *H extracts*, and less than 15% of the ~7 kDa A β species (Fig. 4d and Table 3). In contrast, in *H2 extracts*, the levels of ~4 and ~7 kDa A β were comparable to those measured in *H extracts* (Fig. 4d, Table 4 and Supplementary Fig. 5e–h).

Prior work using *H extracts* indicates that the bulk of the ~4 and ~7 kDa A β detected on Western blots are derived from SDS-labile higher molecular weight assemblies, such that when IP/WB is used it is not possible to differentiate between native low molecular weight ~4 and 7 kDa A β versus ~4 and ~7 kDa A β derived from the breakdown of large assemblies [29]. Nonetheless, our ELISA and IP/WB results demonstrate that relative to *H* and *H2 extracts*, *S extracts* contain much lower levels of both A β monomers and aggregates, but all 3 extracts contain ~4 and ~7 kDa A β species most of which are (probably) derived from SDS-labile higher molecular weight assemblies (Figs. 3 and 4).

S extracts* contain relatively higher levels of low molecular weight A β than *H extracts

To investigate whether the size of A β aggregates in *S* and *H extracts* differed we combined the use of non-denaturing SEC and our highly sensitive A β 42 immunoassay. Since SEC and subsequent analysis of fractions by immunoassays is highly labor-intensive, we chose to examine representative *H* and *S extracts* from 3 of the 9 AD brains. To maximize detection of A β , extracts were chromatographed on a Superdex 200 10/300 GL column eluted in a volatile buffer so fractions could be freeze-dried. Lyophilizates were treated with 5 M GuHCl to disassemble aggregates and render the component monomers measurable using the monomer-preferring A β x-42 immunoassay.

In the 3 cases analyzed, quantifiable levels of A β x-42 were recovered in all 24 fractions from *H extracts* (Supplementary Fig. 6a, c and e). Analysis of fractions from *S extracts* revealed much lower levels of A β x-42, but in all cases A β x-42 was detected in at least fractions –1 to 20 (Supplementary Fig. 6b, d and f). Thus, the size of A β species detected in both *S* and *H extracts* spans a very broad range. Fractions –1 to 2 corresponds to the volume in which blue dextran elutes and before the elution of thyroglobulin (670 kDa).

Consequently, we refer to A β that elutes in fractions –1 to 2 as high molecular weight (HMW). Typically, synthetic A β monomers and dimers elute in fractions 16–18 and 14–15, respectively [29]. Thus, we refer to A β that elutes in or after fraction 12 as low molecular weight (LMW), while A β species that elute in fractions 3–11 are referred to as intermediate molecular weight (IMW). Overall the elution profile of *H* and *S extracts* was similar with 2 prominent peaks, one at HMW and the other at LMW and a trail in between (Fig. 5 and Supplementary Fig. 6). In accord with our other measures of A β (Figs. 3 and 4), there was always considerably less A β in every SEC fraction of *S extracts* when compared to the corresponding fractions from *H extracts* (Supplementary Fig. 6). However, the relative amounts of different sized A β species differed between *H* and *S extracts*. *H extracts* contained relatively more HMW A β , and *S extracts* contained relatively more LMW A β . *S extracts* also tended to contain relatively higher levels of IMW A β than *H extracts* (Fig. 5). These results are consistent with the notion that LMW A β species more readily diffuse from

tissue into buffer than HMW A β , and agree well with the fact that **H extracts** contain more oligomers (Fig. 3e) and GuHCl-sensitive aggregates (Fig. 3b and d) than **S extracts**.

Diffusible forms of A β from Alzheimer's disease brain induce neuritotoxicity on iPSC-derived neurons

In prior studies, we showed that A β extracted from AD brain can disrupt the microtubule cytoskeleton of primary rat hippocampal neurons and cause time-dependent neuritic degeneration [17]. Very recently we employed a live-cell imaging paradigm to measure the effects of AD brain extracts on human neurons [16]. Here we used this new paradigm to assess the activity of **H**, **S** and **H2 extracts**. iPSC-derived neurons (iNs) (Supplementary Fig. 1) were exposed to extracts and imaged every 2 hours for a total of 84 hours. **H** and **S extracts** from AD9 (AD9-H, middle panel; AD9-S, right panel; Fig. 6a) caused a time-dependent decrease in neurite length (**H**, red; **S**, green; $p < 0.001$, **H** vs. control; $p < 0.001$, **S** vs. control; One-Way ANOVA), whereas the **H2 extracts** had no effect on neuritic length (Fig. 6b and c).

Very similar results were obtained with **H**, **S** and **H2 extracts** of AD8 (**H**, red; **S**, green; **H2**, orange; $p < 0.001$, **H** vs. control; $p < 0.001$, **S** vs. control; One-Way ANOVA) (Fig. 6d and Supplementary Fig. 7a) and AD4 (**H**, red; **S**, green; **H2**, orange; $p < 0.001$, **H** vs. control; $p < 0.001$, **S** vs. control; One-Way ANOVA) (Fig. 6e and Supplementary Fig. 7b). Consistent with the reduction in neurite length and branch points detected by IncuCyte, β -tubulin staining revealed a loss of neuritic continuity in neurons treated with **H** and **S**, but not in neurons treated with **H2** (Supplementary Fig. 8).

To examine whether the effects of **H** and **S extracts** were mediated by A β , we conducted additional experiments using AD brain extracts immunodepleted of A β (Fig. 7), and the **H extract** from a control brain (Supplementary Fig. 9a and b). As in our initial experiments, **H** and **S extracts** from AD9 caused a time-dependent decrease in neurite length (**H**, red; **S**, green; $p < 0.001$, **H** vs. control; $p < 0.001$, **S** vs. control; One-Way ANOVA) (Fig. 7a and b), whereas the reduction in neurite length caused by **H** and **S extracts** was prevented by immunodepleted with AW7 (H-ID, light red; S-ID, light green; $p < 0.001$, **H** vs. H-ID; $p < 0.001$, **S** vs. S-ID; One-Way ANOVA) (Fig. 7b and c).

Our finding that **H2 extracts** are inactive implies that **S** and **H extracts** contain equivalent levels of bioactive A β , even though **H extracts** contain more total A β . Based on this assumption, we reasoned that diluting **H extract** to match the A β content of **S extract** would result in lower activity. Consequently, we diluted the **H extract** of AD9 so the levels of A β ₄₂ measured by immunoassay in sample treated with GuHCl would be similar to the corresponding **S extract** (Fig. 7c). Diluted **H extract** did not alter the length of neurites (D, blue; $p < 0.001$, **H** vs. D; $p < 0.001$, **S** vs. D; One-Way ANOVA) (Fig. 7a and b). This finding is in accord with the results that **H2 extracts** are inactive (Fig. 6b and c) and indicate that the specific activity of A β in the AD9 **S extract** is higher than that in the AD9 **H extract**.

Analysis of **H** and **S extracts** of AD8 and AD4 yielded results highly similar to those seen with AD9 extracts (Fig. 7d–i). In each case, ID with AW7 prevented the reduction of neurite length caused by **H** and **S extracts** (AD8: H-ID, light red; S-ID, light green; $p < 0.001$, **H** vs.

H-ID; $p < 0.001$, *S* vs. S-ID; One-Way ANOVA) (AD4: H-ID, light red; S-ID, light green; $p < 0.001$, *H* vs. H-ID; $p < 0.01$, *S* vs. S-ID; One-Way ANOVA) (Fig. 7d, e, g and h), and upon dilution the *H extracts* of AD8 and AD4 (Fig. 7f and i) had little or no effect on neurite length (AD8: D, blue; $p > 0.05$, D vs. control; $p < 0.001$, *H* vs. D; $p < 0.001$, *S* vs. D; One-Way ANOVA) (AD4: D, blue; $p > 0.05$, D vs. control; $p < 0.001$, *H* vs. D; $p < 0.001$, *S* vs. D; One-Way ANOVA) (Fig. 7d, e, g and h). These findings strongly argue that the activity of *H extracts* is attributable to diffusible forms of A β also present in *S extracts*. The fact that diluted *H extracts* are inactive or have greatly reduced activity, but have A β levels comparable or higher than bioactive *S extracts* further supports a preeminent role for diffusible A β and demonstrates that not all A β aggregates have equal bioactivity.

Given that *S extracts* contain less A β than *H extracts*, it is reasonable to ask whether other entities beyond A β might be the real mediators of neurite-disrupting effects evinced by these extracts? The basis for attributing the toxic activity of *H* and *S extracts* to A β relied on the use of the pan anti-A β antibody, AW7 [9, 54]. However, recent evidence suggests that a variety of APP fragments which contain all or part of the A β sequence may have disease relevant activity [56, 57]. Thus, it is conceivable that treatment of extracts with AW7 might not only remove A β , but also bioactive APP fragments. To address this issue, we performed a number of experiments. First, we tested whether AW7 could immunoprecipitate (IP) A η peptides and N-terminally extended (NTE)-A β from cell culture medium rich in these species [56, 57]. In accord with our prior studies, AW7 effectively IP'd A β and NTE-A β [56], but did not precipitate A η (Supplementary Fig. 4). Moreover, antibodies specific for A η failed to detect these species in AW7 IP's of brain extracts, nor as reported previously [29] was there any detectable NTE-A β (Supplementary Fig. 4). Further evidence implicating A β rather than other APP fragments, and in particular soluble A β aggregates, as the proximate toxins in brain extracts comes from our bioactivity studies. Recently, we demonstrated that 3 different anti-A β mAbs can attenuate the neurotoxicity induced by *H extracts* [16] and that the oligomer-preferring mAb 1C22 [16, 28] can prevent toxicity mediated by *S extracts* (data not shown).

Diffusible forms of A β from Alzheimer's disease brain disrupt synaptic plasticity

Deficits in hippocampus-dependent episodic memory are an early feature of AD and much research has focused on testing the idea that rogue soluble forms of A β disrupt the plasticity mechanisms underlying hippocampal memory function [21, 51]. In earlier studies, we found that A β -containing aqueous extracts (analogous to the *H extracts* used here) potentially blocked hippocampal long-term potentiation (LTP) [1, 9, 42, 54]. Here, we investigated the effects of *H* and *S extracts* on LTP.

Initially equal volume of *S* and *H extracts* from AD9 (unadjusted for their A β content) were applied to hippocampal brain slices and their effects on LTP examined. As in prior studies, the A β dependence of these effects was inferred by immunodepletion with AW7. Therefore, a portion of *H* and *S extracts* was immunodepleted (ID) using AW7, or mock immunodepleted with pre-immune rabbit serum (mock ID). For slices that received vehicle (aCSF-B), theta-burst stimulation (TBS) induced strong potentiation that lasted the whole recording period (aCSF, black, $181.53 \pm 5.37\%$, $n = 16$) (Fig. 8a and b) and ID of AD9 *H*

extract allowed a similar response (ID, pink, $190.46 \pm 8.14\%$, $n = 10$; $p > 0.05$, ID vs. aCSF; One-Way ANOVA) (Fig. 8a and b). Consistent with prior reports [9, 42, 54], application of the AD9 *H extract*, but not a *H extract* from a control brain (Supplementary Fig. 9c and d) significantly decreased LTP compared to both the aCSF-B control and ID treatment (*H*, red, $134.27 \pm 5.41\%$, $n = 9$; $p < 0.001$, *H* vs. aCSF; $p < 0.001$, *H* vs. ID; One-Way ANOVA) (Fig. 8a and b). Importantly, the *S extract* of AD9 also potentially blocked LTP (*S*, green, $131.29 \pm 5.07\%$, $n = 8$; $p < 0.001$, *S* vs. aCSF; One-Way ANOVA) (Fig. 8a and b). These results demonstrate that although the *S extract* of AD9, contained much lower levels of A β than the corresponding *H extract*, it nonetheless blocked LTP. Moreover, like the AD9 *H extract*, the block of LTP mediated by AD9 *S extract* was prevented by immunodepletion with AW7 (Supplementary Fig. 10). That is, application of the AD9 *S extract* significantly decreased LTP compared to both the aCSF-B control and ID of AD9 *S extract* (*S*, green, $156.31 \pm 3.95\%$, $n = 5$; $p < 0.05$, *S* vs. aCSF control; $p < 0.001$, *S* vs. ID-S; One-Way ANOVA).

To probe the relative specific activity of A β in the *H* and *S extracts*, we again tested the effects of *H extracts* diluted to match the A β concentration found in *S extracts*. Intriguingly, although diluted *H extract* contained slightly higher levels of A β 42 than *S extract* (Fig. 8c), it did not block LTP (D, blue, $184.2 \pm 9.01\%$, $n = 5$; $p > 0.05$, D vs. aCSF control; $p < 0.001$, D vs. *H*; One-Way ANOVA) (Fig. 8a and b). This result indicates that the *H extract* of AD9 did not contain more active species than the *S extract* of AD9.

To determine whether *S extracts* from other AD brains also contained plasticity-disrupting activity and whether the specific activity of A β (i.e. the extent of LTP disruption caused per unit of A β) in *S extracts* was higher than their corresponding *H extracts* we tested extracts from two additional brains, AD8 and AD4. *H extract* of AD8 significantly blocked LTP (*H*, red, $128.57 \pm 5.13\%$, $n = 12$; $p < 0.001$, *H* vs. aCSF; One-Way ANOVA) (Fig. 8d and e), whereas, ID of AD8 reversed the effects of *H extract* on LTP (ID, pink, $179.79 \pm 10.47\%$, $n = 9$; $p > 0.05$, ID vs. aCSF; $p < 0.001$, ID vs. *H*; One-Way ANOVA) (Fig. 8d and e). As with AD9, the *S extract* of AD8 contained much less A β than the *H extract* of AD8 (Figs. 3–5), but nonetheless the *S extract* of AD8 suppressed LTP (*S*, green, $134.08 \pm 4.06\%$, $n = 12$; $p > 0.05$, *S* vs. *H*, One-Way ANOVA) (Fig. 8d and e). When diluted so the *H extract* of AD8 contained a comparable amount of A β 42 to the corresponding *S extract* (Fig. 8f), the diluted extract only very weakly impaired LTP (D, blue, $160.22 \pm 8.79\%$, $n = 6$; $p < 0.05$, D vs. aCSF, One-Way ANOVA) (Fig. 8d and e). Similar results were seen with extracts of AD4. The *H extract* blocked LTP (*H*, red, $128.65 \pm 4.59\%$, $n = 7$; $p < 0.001$, *H* vs. aCSF, One-Way ANOVA) (Fig. 8g and h), whereas the immunodepleted *H extract* of AD4 did not suppress LTP (ID, pink, $189.74 \pm 6.87\%$, $n = 8$; $p > 0.05$, ID vs. aCSF; $p < 0.001$, ID vs. *H*; One-Way ANOVA) (Fig. 8g and h). Despite the fact that the *S extract* of AD4 contained much less A β than the *H extract* (Figs. 3–5), the *S extract* potentially blocked LTP (*H*, green, $133.87 \pm 6.63\%$, $n = 7$; $p > 0.05$, *S* vs. *H*, One-Way ANOVA) (Fig. 8g and h). Upon dilution, the AD4 *H extract* no longer impaired LTP (D, blue, $171.13 \pm 6.78\%$, $n = 6$; $p > 0.05$, D vs. aCSF, One-Way ANOVA) (Fig. 8g–i), i.e. the specific activity of A β in *S extracts* is greater than in *H extracts*. These findings are completely congruent with the results of our live-neuron imaging experiments (Figs. 6 and 7) and strongly suggest that the A β species responsible for the block of LTP mediated by both the *S* and *H extracts* is a freely diffusible

form of A β , and that a large amount of A β released by mechanical disruption of tissue is relatively inert, at least with respect to impairment of synaptic plasticity.

Discussion

Given the widespread belief that soluble A β oligomers play a central role in AD, it is surprising that only limited efforts have been made to identify toxic A β oligomers from human brain [4, 27, 29, 30, 33, 34, 42]. Use of antemortem CSF might be ideal for this purpose, however, volumes of CSF are limited and prohibit the large scale experiments necessary to isolate and identify toxic forms of A β . In the absence of sufficient CSF we developed a gentle procedure to harvest the diffusible fraction of A β from human cortex. We compared AD-relevant bioactivity and A β content of brain extracts prepared by our new gentle “soaking” technique versus traditional crude homogenization. Remarkably, the levels of A β released by our new method are substantially lower than those obtained by traditional homogenization. This finding suggests that most of the A β found in clarified homogenates (which we refer to as ***H extracts***) arise from mechanical disruption of A β deposits. While prior studies of “soluble A β ” have relied on one or two methods to measure soluble A β aggregates [8, 42], we employed a battery of assays comprising IP/WB, 3 distinct immunoassays, and an SEC/immunoassay approach. Using these diverse methods, we find that both the gently-soaked samples (which we refer to as ***S extracts***) and the traditional ***H extracts*** contain a broad size range of A β species spanning from A β monomers to HMW aggregates. In keeping with prior studies HMW aggregates were the most prominent species in ***H extracts*** [29, 39, 42, 49, 59]. HMW A β aggregates were also a major species in the ***S extracts***; however, compared to their level in ***H extracts***, LMW A β species were more prominent in ***S extracts***. This observation is consistent with the idea that small molecules diffuse more readily than large ones and indicates that the ***S extract*** more accurately represents the freely diffusible forms of A β present in human brain.

Crucially, despite the fact that ***S extracts*** contained much less A β than ***H extracts***, both extracts exhibited comparable levels of bioactivity. In recent work, we have found that ***H extracts*** cause a time- and dose-dependent loss of neuritic length and complexity [16], and here we show that ***S extracts*** have a similar neurotoxic effect. Surprisingly, ***H2 extracts*** which contained high amounts of A β aggregates lacked detectable activity. Moreover, dilution of ***H extracts*** so that their A β content matched that of the corresponding ***S extracts***, led to a loss of neurotoxicity. Together, these results imply that the toxic activity present in ***H extracts*** is attributable to A β emerging from diffusion (i.e. the ***S extract***) and that A β released by mechanical disruption (e.g. ***H2 extract***) does not contribute significantly to this activity.

As with our live-cell imaging studies, dilution of ***H extracts*** led to loss of plasticity-disrupting activity. Thus, the A β in ***H extracts*** that mediates disruption of both neuronal form (neurite integrity) and function (LTP) constitutes only a fraction of the total A β in ***H extracts*** – a diffusible fraction with extremely high “specific activity”.

Prior studies of A β extracted from human brain understandably focused on the forms of A β that are most readily detected [7, 30, 37]. However, our results suggest that the majority of

extractable A β aggregates lack significant bioactivity. This finding complicates both the study of bioactive A β and its therapeutic targeting. For instance, in the 9 AD brains examined here, the total A β levels in *S extracts* were always less than 25 ng/ml (Fig. 3 and Supplementary Tables 1–3), suggesting that methods such as Western blotting (which typically have sensitivity of ~1 ng/gel well) are of limited use to detect and characterize active species in *S extracts*. The rationale for developing putatively “oligomer-specific” therapeutic antibodies is based on the hypothesis that both A β monomers and insoluble fibrillar plaques are relatively innocuous; therefore, an ideal antibody would react weakly with monomers and amyloid plaques, but strongly with soluble aggregates [24]. However, the use of pan-oligomer antibodies is less appealing if only a small portion of soluble aggregates are bioactive and the majority are inert. In this sense, our results may help explain why it has been so difficult to target A β effectively [12, 13].

While it is clear that bioactive A β constitutes only a small fraction of the extractable A β protein, it is uncertain how much of the A β present in *S extracts* is active and it is conceivable that even in *S extracts* only a fraction of the A β may be toxic. Further studies will be required to address this issue and it will be important to determine whether the levels of A β in *S extracts* are elevated in cognitively impaired versus cognitively intact individuals who died with high amyloid burden [53].

Using our native SEC/immunoassay paradigm we discerned no difference in the size distribution of A β species present in the *S* and *H extracts* that might explain their very different specific activities. However, this approach is limited by the low levels of A β in *S extracts* and the species detectable by our x-42 immunoassay. We and others have detected a ~7 kDa A β -immunoreactive species by Western blot of aqueous AD extracts [6, 10, 29, 30, 33, 42], yet this material is not detected by conventional ELISAs [6, 10, 29]. In prior studies we have shown that the ~7 kDa A β detected by Western blot most often arises from the disassembly of SDS-labile HMW assemblies, but that in certain brain samples a small amount of native LMW ~7 kDa A β is detectable [29, 42]. Specifically, when *H extracts* from 33 AD brains were fractionated by SEC only 10 samples contained Western blot detectable levels of ~7 kDa A β which eluted at low molecular weight, but all 33 *H extracts* contained native HMW A β which migrated on denaturing SDS-PAGE at ~7 and 4 kDa [29]. Furthermore, when *H extracts* were treated with strong denaturants and the resulting LMW ~7 kDa A β was isolated by SEC, this material induced aberrant tau phosphorylation and neuritic dystrophy, and impaired LTP [17, 42, 59]. Similarly, solubilization of amyloid plaques by formic acid also liberates a ~7 kDa bioactive A β species [37, 42] and in very recent mass spec studies we detected covalently cross-linked dimers in the ~7 kDa fraction of formic acid-solubilized plaques (Brinkmalm, WH and DMW, unpublished data). As yet the concentration of ~7 kDa A β needed to impair LTP or induce neurotoxicity has not been defined, but it will be important to address this and develop sensitive assays to measure ~7 kDa A β and determine if *S* and *H extracts* contain comparable levels of this species. We believe that ~7 kDa A β is a prime candidate toxin in AD brain, but it is unlikely that only one A β species may prove to be toxic and future studies should deploy antibodies which reportedly recognize sub-groups of oligomers [11, 26] and determine if these antibodies prevent the toxicity mediated by *S* and *H extracts*.

While at this time we cannot identify the precise attributes which imbue toxic activity, our findings have put the focus on low-abundance truly diffusible forms of A β . The use of *S extracts*, first described here, and our relatively high-throughput neuritotoxicity assay together provide a key translational tool that should hasten the decades long quest to definitively identify the most noxious forms of A β present in human brain and enable the development of agents to track and target these entities.

Supplementary Material

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Fig. 1. Methods to extract water-soluble A β from human brain tissue.

Cortical gray matter tissue (~2 g) was cut into small chunks using a McIlwain tissue chopper (set at 0.5 mm). The diced tissue was mixed and divided into halves. One portion was homogenized in 5 vol. of aCSF-B with 25 strokes of a Teflon-glass Dounce homogenizer. The homogenate was then centrifuged at 200,000 g and 4°C for 110 minutes. The upper 80% of the supernatant was removed and designated as **H extract**. The other portion of tissue was incubated in 5 vol. of aCSF-B at 4°C for 30 minutes with gentle side-to-side mixing. To separate tissue from the aCSF-B into which biomolecules had diffused, and to minimize mechanical disruption of tissue, the suspension was centrifuged at low speed (2,000 g and 4°C for 10 minutes). The upper 90% of the supernatant was removed and this material centrifuged as for **H extract**. The upper 90% of this second supernatant, designated as **S extract**, was removed. **H2 extracts** were prepared using the pellets generated when preparing **S extracts**. The 2,000 g and 200,000 g pellets were pooled and Dounce homogenized in 5 volumes of ice-cold aCSF-B, centrifuged at 200,000 g for 110 minutes and 4°C. The upper 80% of supernatant was removed and designated as **H2 extract**.

Fig. 2. Similar amounts of water-soluble proteins are detected in *S* extracts and *H* extracts while lower levels of soluble protein are detected in *H2* extracts.

Extracts from a total of 10 brains (9 from patients with AD, and 1 from a control free of AD) were prepared as outlined in Fig. 1 and protein content measured using a BCA assay (a). *H* extracts are indicated by red colored bars and red lettering; *S* extracts are in green and *H2* extracts are in orange. *H* and *S* extracts from the same brains contain similar levels of total protein, while *H2* extracts contain lower levels. Western blotting of equal volumes of brain extracts revealed similar levels of sAPP (b) and BDNF (c) in *H* and *S* extracts from the same brains, but much lower in *H2* extracts. The relative intensity of protein bands was determined using LiCOR software and these values were used to estimate the percentage of sAPP and BDNF in *S* or *H2* relative to *H*, i.e. the $S/H \times 100$ or $H2/H \times 100$ (d). All values are based on duplicated measurements from the same Western blot and are representative of at least 2 independent experiments. Black asterisks indicate samples used in subsequent bioactivity studies. Full-length blots are shown in Supplementary Fig. 3.

Fig. 3. The levels of different forms of Aβ are significant lower in *S* extracts than *H* or *H2* extracts.

Extracts of the same 10 brains shown in Fig. 2 were analyzed for 5 distinct forms of Aβ using 3 different MSD-based immunoassays. Only results for the brain extracts used in subsequent bioactivity studies are shown, but the data for all the 10 brains are listed in Supplementary Tables 1–3. The Aβx-40 and Aβx-42 assays preferentially detect Aβ monomers ending at Val40 (a) and Ile 42 (c), respectively. Unmanipulated *H*, *S* and *H2* extracts are shown in open red bars, open green bars and open orange bars, respectively.

Incubation of samples with GuHCl dissociates soluble A β aggregates allowing increased detection of monomer by the A β x-40 (**b**) and A β x-42 (**d**) assays. **H**, **S** and **H2 extracts** pre-incubated with GuHCl are shown in filled red bars, filled green bars and filled orange bars, respectively. The oligomer assay preferentially detects soluble aggregates of various A β sequences and measured higher levels of soluble aggregates in **H extracts** (open red bars) and **H2 extracts** (open orange bars) than **S extracts** (open green bars) (**e**). Individual bars are the average $\pm$ SD of each sample analyzed in triplicate. When error bars are not visible, they are smaller than the size of the symbol. For all 10 brains, the percentage of A β in **S** or **H2 extracts** relative to **H extracts** is shown as $S/H \times 100$ or $H2/H \times 100$ (**f**). G- and G+ denote samples treated without and with GuHCl.

Fig. 4. *H*, *S* and *H2* extracts contain SDS-stable ~4 and ~7 kDa Aβ, but the levels are much lower in *S* extracts than *H* or *H2* extracts.

Equal volumes of the same extracts analyzed in Figs. 2 and 3 were used for immunoprecipitation/Western blotting (IP/WB). Only results for AD4, AD9 and C1 are shown here, but all IP/WBs for the other brains are shown in Supplementary Fig. 5 (a-c). Samples were IP'd with either anti-Aβ antiserum, AW7, or pre-immune serum (PI) and WB was performed using the anti-Aβ40 and anti-Aβ42 antibodies, 2G3 and 21F12. Five ng of synthetic Aβ1-42 was loaded on each gel to allow comparison between gels. AD brain numbers, the types of extract used (*H*, red; *S*, green; *H2*, orange) 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 Aβ monomer and double arrow refers to the SDS-stable ~7 kDa Aβ species. Non-specific bands detected when PI was used are indicated

by a solid black line. From the results shown here and in Supplementary Fig. 5, the relative amount of both ~4 and 7 kDa A β estimated using LiCOR software in *S extracts* was always less than 40% of that detected in the corresponding *H extracts*, whereas the relative amount of ~4 and 7 kDa A β was always greater than 50% in *H2 extracts* (d).

Fig. 5. *S* extracts contain relatively higher levels of low molecular weight Aβ than *H* extracts. Extracts from brains AD9 (a), AD8 (b) and AD4 (c) were fractionated using a Superdex 200 size exclusion column eluted with 50 mM ammonium bicarbonate, pH 8.5. Fractions were lyophilized, denatured with GuHCl and analyzed using the MSD-based x-42 assay. Values are normalized based on the total amount of Aβ_{x-42} detected over an entire 24 fraction chromatogram, i.e. fractions -2 to 21. *H* extracts are shown with red symbols and lines, and *S* extracts are in green. *S* extracts contain relatively higher levels of low molecular weight Aβ than *H* extracts, and *H* extracts contain relatively higher levels of high molecular weight Aβ than *S* extracts. Elution of globular standards is indicated by downward pointing arrows labeled 17, 44, 158, and 670 (in kDa). Fraction 0 indicates the peak fraction in which Blue dextran eluted.

Fig. 6. *S* and *H* extracts produce comparable neuritotoxicity, while *H2* extracts exert no toxicity. Live-cell imaging was used to monitor the effect of AD brain extracts on iPSC-derived neurons (iNs). On post-induction day 21, iNs were treated with medium alone (Control, black) or AD extract (AD9-H, red; AD9-S, green) and cells imaged for 84 hours (a). Phase contrast images (top panel) at 0 and 84 hours were analyzed using the IncuCyte NeuroTrack algorithm to identify neurites (middle panel). Identified neurites (pink) are shown superimposed on the phase contrast image (bottom panel). Scale bars are 100 μ m. Each well of iNs was imaged for 6 hours prior to addition of sample and NeuroTrack-identified neurite

length used to normalize neurite length measured at each interval after addition of sample. **H**, **S** and **H2 extracts** were tested for their effects on iNs at 1:4 dilution. The values shown in graphs are the average of triplicate wells for each treatment $\pm$ SD. Time course plots (**b**) show that **H extract** (red) and **S extract** (green) cause neuritotoxicity when compared to control (black), while **H2 extract** (orange) does not show any toxicity. (**c-e**) Histogram plots of normalized neurite length (mean values $\pm$ SD) are derived from the last 6 hours of the traces shown in **b** (AD9) and in Supplementary Fig. 7a and b (AD8 and AD4). The results shown are representative of three independent experiments. Treatments were examined by one-way ANOVA and significant differences are denoted as *** $p < 0.001$; n.s. indicates not significant.

Fig. 7. *S* extracts contain less Aβ than *H* extracts yet more potently induce neuritotoxicity than *H* extracts diluted to match the Aβ content of *S* extracts.

H and *S* extracts, as well as H-ID, S-ID and D samples, were tested for their effects on iNs (a, b, d, e, g and h). H-ID and S-ID denote *H* extract and *S* extract from which Aβ was immunodepleted using AW7. All samples were tested at 1:4 dilution. D denotes *H* extracts that were diluted to match the Aβ_{x-42} content in corresponding *S* extracts. The values shown in graphs are the average of triplicate wells for each treatment ± SD. (b, e and h) Histogram plots of normalized neurite length are derived from the last 6 hours of the traces shown in a, d and g, and are presented as mean values ± SD. The results shown are representative of at least three independent experiments. Treatments were examined by one-way ANOVA and significant differences are denoted as ** p<0.01 and *** p<0.001. The Aβ content in extracts from AD9, AD8 and AD4 were determined by Aβ_{x-42} immunoassay plus

pre-treatment with 5 M GuHCl (**c**, **f** and **i**). Individual bars are the average $\pm$ SD of each sample analyzed in triplicate. Data are representative of at least 2 experiments.

Fig. 8. *S* extracts contain less Aβ than *H* extracts yet more potently block LTP than *H* extracts diluted to match the Aβ content of *S* extracts.

H and *S* extracts, as well as immunodepleted and diluted *H* extracts, were tested for their effects on LTP (a, b, d, e, g and h). ID denotes *H* extract from which Aβ was immunodepleted using AW7. D denotes *H* extracts that was diluted to match the Aβ_{x-42} content in *S* extract. Time course plots (a, d and g) show that *H* extracts (red) and *S* extracts (green) block LTP, when compared to aCSF control (black). ID samples (pink) do not block LTP. Similarly, D samples (blue) either do not inhibit LTP (AD9 and AD4) or cause only a modest decrement of LTP (AD8). The horizontal bar represents the time during which the vehicle or extract was present in the recording solution. ↑TBS indicates theta burst

stimulation used to induce LTP. The slopes of fEPSPs are shown as mean $\pm$ SD relative to baseline. Histogram plots (**b**, **e** and **h**) show the average potentiation for the last 10 min of the traces in **a**, **d** and **g**. The effect of treatments relative to aCSF vehicle were examined by one-way ANOVA and significant differences are denoted as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. The A β content in extracts from AD9, AD8 and AD4 were determined by A β x-42 immunoassay $\pm$ pre-treatment with 5 M GuHCl (**c**, **f** and **i**). Individual bars are the average $\pm$ SD of each sample analyzed in triplicate. A β measurements are representative of at least 2 experiments.

Table 1

Antibodies used in this study and their sources

Antibody	Type	Epitope	Dilution for IP	Conc. for WB	Conc. for ELISA	Conc. for ICC	Source/Reference
22C11	Monoclonal	APP66-81	-	1 µg/ml	-	-	Millipore/[38]
2E9	Monoclonal	APP545-555	-	1 µg/ml	-	-	Haass Lab/[57]
3D6	Monoclonal	Aβ1-5	-	-	0.4 µg/ml	-	Elan/[18]
6E10	Monoclonal	Aβ6-10	-	1 µg/ml	-	-	Biolegend/[32]
266	Monoclonal	Aβ16-23	-	-	3 µg/ml	-	Elan/[41]
4G8	Monoclonal	Aβ17-24	-	1 µg/ml	-	-	Biolegend/[22]
2G3	Monoclonal	Aβ terminating at Val40	-	1 µg/ml	0.2 µg/ml	-	Elan/[18]
HJ2	Monoclonal	Aβ terminating at Val40	-	1 µg/ml	-	-	Holtzman lab/[20]
21F12	Monoclonal	Aβ terminating at Ala42	-	1 µg/ml	0.4 µg/ml	-	Elan/[18]
AW7 [†]	Polyclonal	Pan anti-Aβ	1:50	-	-	-	Walsh Lab/[31]
1C22	Monoclonal	Aβ aggregates	-	-	3 µg/ml	-	Walsh Lab/[28]
N-20	Polyclonal	Human BDNF	-	1 µg/ml	-	-	Santa Cruz/[46]
MAB5564	Monoclonal	β-tubulin	-	-	-	2 µg/ml	Millipore
K9JA	Polyclonal	Tau (243-441)	-	-	-	2 µg/ml	DAKO

Abbreviations: IP, immunoprecipitation; WB, Western blot; ELISA, enzyme-linked immunosorbent assay; Aβ, amyloid beta; APP, amyloid precursor protein.

[†]AW7 is a pan anti-Aβ antiserum which contains antibodies which recognize multiple Aβ epitopes and a range of aggregation states.

Table 2

Demographic details of the cases used in this study

Case	Age	Gender	PMI (hours)	Clinical diagnosis	Neuropathology diagnosis	B&B, CERAD
AD1	83	F	NA	AD	AD	NA, C
AD2	69	F	4	AD	AD	VI, C
AD3	68	F	36	AD	AD	VI, C
AD4 [*]	84	F	9	AD/Mixed dementia	AD	VI, C
AD5	84	F	15	AD	AD	VI, C
AD6	66	F	28	AD/FTD	AD	VI, C
AD7	69	F	16	AD	AD	NA
AD8 [*]	68	F	24	AD	AD	V/VI, NA
AD9 [*]	67	M	28	AD	AD	VI, C
C1	58	F	18	Not demented	Control	II, NA

Abbreviations: AD, Alzheimer's disease; B&B, Braak stage; CERAD, Consortium to establish a registry for AD score; M, male; F, female; PMI, post-mortem interval; NA, information not available; FTD, frontal temporal dementia.

^{*} denotes samples used in subsequent bioactivity assays.

Table 3Protein content of *S extract* relative to *H extract* (*S/H* x 100)

Assays	Total protein	sAPP	BDNF	Monomeric A β x-40	Dissociated A β x-40	Monomeric A β x-42	Dissociated A β x-42	Oligomeric A β	~7 kDa A β	~4 kDa A β
	BCA	WB	WB	ELISA	ELISA	ELISA	ELISA	ELISA	WB	WB
AD1	80.4	63.8	85.7	86.0	51.3	28.6	10.3	18.1	13.4	4.8
AD2	73.2	79.9	89.1	6.0	3.2	26.4	23.2	11.0	10.7	2.5
AD3	90.0	103.9	85.8	58.5	45.2	31.5	16.4	14.1	14.8	3.8
AD4 [*]	83.6	75.2	99.4	6.4	6.7	29.9	7.4	12.6	7.2	0.7
AD5	75.1	95.2	100.4	6.3	11.7	57.5	11.4	9.0	7.9	1.0
AD6	99.0	83.8	94.3	130.5	149.6	37.9	46.9	52.7	31.5	18.1
AD7	109.7	91.2	113.5	51.9	42.5	35.3	18.9	40.4	11.7	3.2
AD8 [*]	85.8	102.8	106.7	48.8	62.9	51.5	27.1	62.4	32.0	18.7
AD9 [*]	78.3	85.0	90.4	13.8	31.8	22.6	13.6	15.1	10.9	1.6
C1	105.0	101.1	100.3	ND	ND	ND	ND	ND	ND	ND
Average	88.0	88.2	96.6	45.4	45.0	35.7	19.5	26.2	15.6	6.0

Abbreviations: ND, not detectable.

^{*} denotes samples used in subsequent bioactivity assays.

Table 4Protein content of *H2 extract* relative to *H extract* (*H2/H* x 100)

	Total protein	sAPP	BDNF	A β x-40 (G-)	A β x-40 (G+)	A β x-42 (G-)	A β x-42 (G+)	oA β	~7 kDa A β	~4 kDa A β
Assays	BCA	WB	WB	ELISA	ELISA	ELISA	ELISA	ELISA	WB	WB
AD1	37.4	41.5	18.4	130.1	60.0	120.7	57.1	63.9	67.6	80.0
AD2	42.8	27.6	25.5	116.8	107.6	147.6	128.5	128.8	125.8	112.5
AD3	44.0	29.8	23.5	130.2	88.2	149.6	87.6	93.4	118.8	134.2
AD4*	43.7	35.9	16.8	186.3	155.5	173.5	109.1	106.5	110.1	104.1
AD5	33.7	39.3	22.6	140.3	152.5	146.6	173.9	122.5	125.4	174.8
AD6	35.3	36.9	18.8	113.1	67.5	100.8	86.3	28.9	73.8	90.4
AD7	32.3	30.4	18.0	150.9	82.2	151.8	77.1	125.3	74.6	117.7
AD8*	38.4	28.0	32.5	78.9	52.1	107.9	88.5	78.1	62.7	53.6
AD9*	46.5	40.3	34.2	153.9	152.2	135.3	149.8	44.7	132.9	174.0
C1	33.7	35.6	7.3	ND	ND	ND	ND	ND	ND	ND
Average	38.8	34.5	21.8	133.4	102.0	137.1	106.4	88.0	99.1	115.7

Abbreviations: ND, not detectable.

* denotes samples used in subsequent bioactivity assays.