

# Monomeric amyloid- $\beta$ reduced amyloid- $\beta$ oligomer-induced synapse damage in neuronal cultures

Clive Bate<sup>a,\*</sup>, Alun Williams<sup>b</sup>

<sup>a</sup> Department of Pathology and Pathogen Biology, Royal Veterinary College, Hawkshead Lane, North Mymms, Herts AL9 7TA, UK

<sup>b</sup> Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK

## ARTICLE INFO

### Keywords:

Amyloid- $\beta$   
Cholesterol  
Monomers  
Oligomers  
Phospholipase A<sub>2</sub>  
Synapses

## ABSTRACT

Alzheimer's disease is a progressive neurodegenerative disease characterized by the accumulation of amyloid- $\beta$  (A $\beta$ ) in the brain. A $\beta$  oligomers are believed to cause synapse damage resulting in the memory deficits that are characteristic of this disease. Since the loss of synaptic proteins in the brain correlates closely with the degree of dementia in Alzheimer's disease, the process of A $\beta$ -induced synapse damage was investigated in cultured neurons by measuring the loss of synaptic proteins. Soluble A $\beta$  oligomers, derived from Alzheimer's-affected brains, caused the loss of cysteine string protein and synaptophysin from neurons. When applied to synaptosomes A $\beta$  oligomers increased cholesterol concentrations and caused aberrant activation of cytoplasmic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>). In contrast, A $\beta$  monomer preparations did not affect cholesterol concentrations or activate synaptic cPLA<sub>2</sub>, nor did they damage synapses. The A $\beta$  oligomer-induced aggregation of cellular prion proteins (PrP<sup>C</sup>) at synapses triggered the activation of cPLA<sub>2</sub> that leads to synapse degeneration. Critically, A $\beta$  monomer preparations did not cause the aggregation of PrP<sup>C</sup>; rather they reduced the A $\beta$  oligomer-induced aggregation of PrP<sup>C</sup>. The presence of A $\beta$  monomer preparations also inhibited the A $\beta$  oligomer-induced increase in cholesterol concentrations and activation of cPLA<sub>2</sub> in synaptosomes and protected neurons against the A $\beta$  oligomer-induced synapse damage. These results support the hypothesis that A $\beta$  monomers are neuroprotective. We hypothesise that synapse damage may result from a pathological A $\beta$  monomer:oligomer ratio rather than the total concentrations of A $\beta$  within the brain.

## 1. Introduction

Alzheimer's disease (AD) is a complex neurological disorder characterized by a progressive dementia resulting from synapse failure (Selkoe, 2002; Tanzi, 2005). The amyloid hypothesis maintains that the pivotal event in AD is the production of amyloid- $\beta$  (A $\beta$ ) peptides following the proteolytic cleavage of the amyloid precursor protein (APP) (De Strooper et al., 2010; Hardy and Selkoe, 2002). The accumulation of C-terminal fragments (A $\beta$ ) within the brain is thought to cause the synapse dysfunction and the memory loss that is characteristic of AD. A $\beta$  has the capacity to self-aggregate, and consequently is found in different forms ranging from monomers and small soluble oligomers to much larger fibrils and plaques. The soluble A $\beta$  oligomers are currently considered to be the principal mediators of synapse damage (Yang et al., 2017).

The mechanisms of Alzheimer's-related synapse damage can be

examined by incubation of cultured neurons with A $\beta$ . Since the loss of synapses and synaptic proteins is a feature of AD that strongly correlates with cognitive decline in AD (Counts et al., 2006; Masliah et al., 1991; Reddy et al., 2005) synapse density was measured by determining the amounts of synaptophysin and cysteine string protein (CSP) in cultured neurons (Lipton et al., 2001). Soluble forms of A $\beta$  derived from the brains Alzheimer's patients caused synapse degeneration in neurons (Yang et al., 2017). The biological effects of these A $\beta$  oligomers occurred at picomolar concentrations, similar concentrations to those found in the cerebrospinal fluid of Alzheimer's patients (Bibl et al., 2007; McLean et al., 1999; Mehta et al., 2000). The role of A $\beta$  monomers that are found in high concentrations in the brain are poorly understood; while some studies report that A $\beta$  monomers are not toxic (Shankar et al., 2007; Walsh et al., 2002) another study suggested that they were neuroprotective (Giuffrida et al., 2009). Confusion may arise due to the heterogeneity of A $\beta$  in monomer preparations, in addition to

**Abbreviations:** AD, Alzheimer's disease; APP, amyloid precursor protein; A $\beta$ , amyloid- $\beta$ ; PrP<sup>C</sup>, cellular prion protein; cPLA<sub>2</sub>, cytoplasmic phospholipase A<sub>2</sub>; CSP, cysteine-string protein; ELISA, enzyme-linked immunoassay; Fab, fragment antigen-binding; GPI, glycosylphosphatidylinositol; mAb, monoclonal antibody; PLAP, phospholipase A<sub>2</sub>-activating peptide; PG, prostaglandin; SDS, sodium dodecyl sulphate; SD, standard deviation; VAMP, vesicle-associated membrane protein

\* Corresponding author.

E-mail addresses: [cbate@rvc.ac.uk](mailto:cbate@rvc.ac.uk) (C. Bate), [aw510@cam.ac.uk](mailto:aw510@cam.ac.uk) (A. Williams).

<https://doi.org/10.1016/j.nbd.2017.12.007>

Received 11 May 2017; Received in revised form 12 November 2017; Accepted 12 December 2017

0969-9961/© 2017 Published by Elsevier Inc.

A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub> peptides there are other APP fragments in cerebrospinal fluid (Brinkmalm et al., 2012) and that some of these carboxy-terminal fragments of APP have biological activity (Willem et al., 2015). In the present study A $\beta$  monomer preparations and A $\beta$  oligomers were isolated from soluble brain extracts and their biological activity studied.

Increasing evidence suggests that aberrant activation of cytoplasmic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) plays an important role in A $\beta$  oligomer-induced synapse damage. For example, A $\beta$  activates cPLA<sub>2</sub> (Anfuso et al., 2004; Shelat et al., 2008) and pharmacological inhibition of cPLA<sub>2</sub> protects cultured neurons against A $\beta$ -induced synapse damage (Bate et al., 2010). In addition, cPLA<sub>2</sub> inhibitors ameliorated cognitive decline in a mouse model of AD (Sanchez-Mejia et al., 2008). Here we demonstrate that whereas A $\beta$  oligomers increased synaptic cholesterol concentrations, activated cPLA<sub>2</sub> and caused synapse damage in cultured neurons, A $\beta$  monomers had none of those effects. The cellular prion protein (PrP<sup>C</sup>) mediates A $\beta$ -induced memory defects (Lauren et al., 2009) and aggregation of PrP<sup>C</sup> by A $\beta$  oligomers caused the activation of cPLA<sub>2</sub> and synapse damage (Bate and Williams, 2011). Here we report that A $\beta$  monomers did not cause aggregation of PrP<sup>C</sup>; rather that they reduced the aggregation of PrP<sup>C</sup> by A $\beta$  oligomers. The presence of A $\beta$  monomers significantly reduced the A $\beta$  oligomer-induced activation of cPLA<sub>2</sub> and synapse damage.

## 2. Materials and methods

### 2.1. Primary neuronal cultures

Cortical neurons were prepared from the brains of day 15.5 mouse embryos as described (Bate et al., 2010). Cells were suspended in Ham's F12 medium containing 5% foetal calf serum and seeded at  $2 \times 10^5$  cells/well in 48 well plates that had been coated with poly-L-lysine. After 2 h, cultures were shaken and washed to remove non-adherent cells. Neurons were grown in neurobasal medium containing B27 components and 5 ng/ml nerve growth factor (Sigma) for 10 days. Immunohistochemistry revealed that approximately 95% of cells were neurofilament positive. All experiments were performed in accordance with European regulations (European Community Council Directive, 1986, 56/609/EEC) and approved by the local authority veterinary service/ethical committee. To determine cell viability they were incubated with 50  $\mu$ M thiazolyl blue tetrazolium bromide for 3 h at 37 °C. The supernatant was removed, the formazan product solubilized in 200  $\mu$ l of dimethyl sulfoxide, transferred to an immunoassay plate and absorbance read at 595 nm. Cell survival was calculated with reference to untreated cells (100% survival). Neurons were incubated with A $\beta$  preparations for 24 h. For other studies, neurons were pre-treated with A $\beta$  monomers or control medium for 1 h and then incubated with A $\beta$  oligomers or a phospholipase A<sub>2</sub>-activating protein (PLAP) (Bachem) for 24 h. Treated neurons were washed 3 times with PBS and homogenized in 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, Nonidet P-40, 0.5% sodium deoxycholate and 0.2% SDS at  $10^6$  cells/ml. Mixed protease inhibitors (4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride, Aprotinin, Leupeptin, Bestatin, Pepstatin A and E-46) and a phosphatase inhibitor cocktail including PP1, PP2A, microcystin LR, cantharidin and p-bromotetramisole (Sigma) were added and nuclei and large fragments were removed by centrifugation ( $1000 \times g$  for 5 min).

### 2.2. Western blotting

Samples were mixed with Laemmli buffer containing  $\beta$ -mercaptoethanol, heated to 95 °C for 5 min and proteins were separated by electrophoresis on 15% polyacrylamide gels. Proteins were transferred onto a Hybond-P polyvinylidene fluoride membrane by semi-dry blotting. Membranes were blocked using 10% milk powder; synapsin-1 was detected with goat polyclonal (Santa Cruz Biotech), synaptophysin with MAB368 (Abcam), cysteine-string protein (CSP), with rabbit

polyclonal anti-CSP ((sc-33154) Santa Cruz), vesicle-associated membrane protein (VAMP)-1 with monoclonal antibody (mAb) 4H302 (Abcam), caveolin with rabbit polyclonal antibodies to caveolin (Upstate). These were visualised using a combination of biotinylated anti-mouse/goat/rat/rabbit IgG (Sigma), extravidin-peroxidase and enhanced chemiluminescence.

### 2.3. Synaptophysin ELISA

The amount of synaptophysin in samples was determined by ELISA as described (Bate et al., 2010). Maxisorb immunoplates (Nunc) were coated with an anti-synaptophysin mAb (MAB368-Chemicon) and blocked with 5% milk powder. Samples were added for 1 h and bound synaptophysin was detected using rabbit polyclonal anti-synaptophysin (Abcam) followed by a biotinylated anti-rabbit IgG (Sigma), extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenol phosphate solution (Sigma). Absorbance was measured at 405 nm. Samples were expressed as “units synaptophysin” where 100 units was the amount of synaptophysin in  $10^6$  untreated cells.

### 2.4. CSP ELISA

Maxisorb immunoplates were coated with a mouse mAb to CSP ((sc-136468) Santa Cruz) and blocked with 5% milk powder. Samples were added for 1 h and bound CSP was detected using rabbit polyclonal anti-CSP ((sc-33154) Santa Cruz) followed by a biotinylated anti-rabbit IgG, extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenol phosphate solution. Absorbance was measured at 405 nm. Samples were expressed as “units CSP” where 100 units was the amount of CSP in  $10^6$  untreated cells.

### 2.5. Isolation of synaptosomes

Synaptosomes were prepared from mouse cortical neuronal cultures, as described above, on a discontinuous Percoll gradient as described (Dunkley et al., 2008). Neurons were homogenized at 4 °C in 1 ml of SED solution (0.32 M sucrose, 5 mM Tris-HCl pH 7.2, 1 mM EDTA, and 0.25 mM dithiothreitol) and centrifuged at  $1000 \times g$  at 4 °C. The supernatant was transferred to a 4-step gradient of 3, 7, 15, and 23% Percoll in SED solution and centrifuged at  $16,000 \times g$  for 30 min at 4 °C. The synaptosomes were collected from the interface between the 15% and 23% Percoll and washed ( $16,000 \times g$  for 10 min at 4 °C) and suspended in neurobasal medium containing B27 components at a concentration equivalent to  $5 \times 10^6$  neurons per ml. All synaptosomes were used on the same day of preparation. Synaptosomes were incubated with A $\beta$  monomers or A $\beta$  oligomers for 1 h at 37 °C. In one experiment synaptosomes were pre-treated with A $\beta$  monomers for 30 min and then incubated with A $\beta$  oligomers for 1 h at 37 °C. Treated synaptosomes were washed with ice cold PBS and suspended in ice cold extraction buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.4, 10 mM EDTA, 0.2% SDS and mixed protease/phosphatase inhibitors (as above)).

### 2.6. Synaptic vesicle recycling

The fluorescent dye FM1-43 that is taken up into synaptic recycling vesicles was used as an indicator of synaptic activity (Parodi et al., 2010). Treated synaptosomes were pulsed with 5  $\mu$ M FM1-43 and 1  $\mu$ M ionomycin, a calcium ionophore used to stimulate neurotransmitter release, for 5 min, washed 5 times in ice cold PBS and homogenized in methanol. Soluble extracts were transferred into Sterilin 96 well black microplates and fluorescence was measured using excitation at 480 nm and emission at 625 nm. Samples were expressed as “% fluorescence” where 100% fluorescence was the fluorescence of  $10^6$  control synaptosomes pulsed with FM1-43 and ionomycin.

## 2.7. Cholesterol content

The concentrations of cholesterol in samples were measured using the Amplex Red cholesterol assay kit (Life Technologies) (Robin et al., 2010). Briefly, control and treated synaptosomes were washed ( $400 \times g$ , 10 min) and homogenized in 500  $\mu$ l isopropanol:NP40 (9:1, v/v) and sonicated in a waterbath (30 min). Samples were centrifuged ( $10,000 \times g$ , 1 min); the supernatants pre-treated with catalase before the enzyme cocktail of the Amplex-Red kit was added (0.1 M potassium phosphate buffer, pH 7.4; 0.25 M NaCl, 5 mM cholic acid, 0.1% Triton X-100, cholesterol oxidase, horse radish peroxidase and 0.4 mM 10-acetyl-3,7-dihydroxyphenoxazine ( $\pm$  cholesterol esterase)) were added and incubated at 37 °C for 30 min. Cholesterol is oxidised by cholesterol oxidase to yield hydrogen peroxide and ketones. The hydrogen peroxide reacts with 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent) to produce highly fluorescent resorufin, which is measured by excitation at 530 nm and emission detection at 590 nm. Each experiment contained a dose-response of cholesterol standards and solvent only controls. Cholesterol concentrations of samples were calculated by reference to the cholesterol standards.

## 2.8. Activated cPLA<sub>2</sub> ELISA/prostaglandin (PGE)<sub>2</sub> ELISA

The activation of cPLA<sub>2</sub> is accompanied by phosphorylation of the 505 serine residue creating an epitope that can be measured by ELISA as described (Bate et al., 2010). Briefly, Maxisorb immunoplates were coated with 0.5  $\mu$ g/ml of the mouse mAb anti-cPLA<sub>2</sub>, clone CH-7 (Upstate) and blocked with 5% milk powder. Samples were incubated for 1 h and the amount of bound activated cPLA<sub>2</sub> was detected using a rabbit polyclonal anti-phospho-cPLA<sub>2</sub> (Cell Signalling Technology), followed by biotinylated anti-rabbit IgG, extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenyl phosphate solution. Absorbance was measured at 405 nm and the amounts of activated cPLA<sub>2</sub> present were expressed as “units activated cPLA<sub>2</sub>” where 1 unit was defined as the amount of activated cPLA<sub>2</sub> in control samples. The amounts of PGE<sub>2</sub> in synaptosomes were determined using a competitive enzyme immunoassay kit (R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

## 2.9. Brain extracts

Soluble extracts were prepared from the brains (frontal cortex) of Alzheimer's patients (Asterand) using an adapted methodology of that previously described (Shankar et al., 2008). Briefly, brain tissue was cut into small pieces and approximately 100 mg was added to 2 ml tubes containing lysing matrix D beads (Q-Bio). Ice cold 20 mM Tris, pH 7.4 containing 150 mM NaCl was added to an equivalent of 100 mg brain tissue/ml, tubes were shaken for 10 min (Disruptor genie, Scientific Instruments). This process was performed 3 times before tubes were centrifuged at  $16,000 \times g$  for 10 min to remove particulate matter. Soluble material was prepared by passage through a 50 kDa filter (Sartorius) ( $16,000 \times g$  for 30 min to remove proteolytic enzymes, membrane-bound and larger forms of A $\beta$ ). The remaining material was then desalted (3 kDa filter (Sartorius)) to eliminate bioactive small molecules and drugs and the retained material collected (preparation contains molecules with molecular weights between 3 and 50 kDa). One of the reasons that “natural A $\beta$ ” preparations were used in this study was that natural A $\beta$  oligomers and monomers (unlike synthetic peptides) are stable (Jin et al., 2011; Shankar et al., 2008; Walsh et al., 2002). For example, after A $\beta$  monomers had been incubated for 7 days at 37 °C in a cell free system we did not find any A $\beta$  oligomers. Brain extracts were not pooled, rather monomers and oligomers were isolated from 3 main brain extracts and each was measured in triplicate.

## 2.10. Monomer and oligomer preparations

Monomer preparations were prepared by passage through a 10 kDa filter (Sartorius). In theory A $\beta$  dimers have a molecular weight of 9 kDa and should pass through the 10 kDa filter. However, natural A $\beta$  is bound to membrane components that increase the molecular weight of A $\beta$  dimers and consequently no A $\beta$  dimers were detected in 10 kDa filtrates. It should be noted that A $\beta$  monomer preparations contained A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub> (confirmed by end-specific ELISA, see below) and other small APP fragments. Oligomer preparations were collected from the retained material following a 10 kDa filtration (10 to 50 kDa). For monomer-depleted brain extracts 1 ml samples were centrifuged in a 10 kDa filter and the retained material diluted back to 1 ml with culture medium.

Size exclusion chromatography was performed on samples using a Superdex 75 PC column (separates peptides ranging from 3 kDa to 70 kDa) (GE Healthcare) with an elution rate of 0.2 ml/min. Preparations were stored at –80 °C. For cell experiments preparations were diluted in neurobasal medium containing B27 components. For immunoblots, preparations were mixed with an equal volume of 0.5% NP-40, 5 mM CHAPS, 50 mM Tris, pH 7.4 and separated by gel electrophoresis. Proteins were transferred onto a Hybond-P polyvinylidene fluoride membrane by semi-dry blotting and blocked using 10% milk powder. A $\beta$  was detected by incubation with mAb 6E10, reactive with amino acids 1–16 of human A $\beta$  (Covance), biotinylated anti-mouse IgG, extravidin-peroxidase and enhanced chemiluminescence.

## 2.11. Immunodepletions

Brain extracts or monomer preparations were incubated with 1  $\mu$ g/ml mAb 6E10 (reactive with amino acids 1–16, Covance) or an isotype control (mock-depletion) and incubated at 4 °C on rollers for 24 h. Protein G microbeads were added (10  $\mu$ l/ml) (Sigma) for 2 h and protein G bound-antibody complexes removed by centrifugation. The depleted media was passed through a 50 kDa filter before further use.

## 2.12. Sample preparation for A $\beta$ <sub>40</sub>/A $\beta$ <sub>42</sub> ELISA

To detach A $\beta$ <sub>42</sub> from membrane components that blocked specific epitopes samples (300  $\mu$ l) were mixed with 700  $\mu$ l of propan-2-ol and sonicated. Proteins were precipitated by adding 250  $\mu$ l 100% w/v trichloroacetic acid, incubating on ice for 30 mins and centrifugation ( $16,000 \times g$  for 10 mins at 4 °C). The pellet was washed twice with ice cold acetone, suspended, first in 1% NH<sub>4</sub>OH and then in a buffer containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.2% SDS and sonicated.

## 2.13. A $\beta$ <sub>40</sub>/A $\beta$ <sub>42</sub> ELISA

Nunc Maxisorb immunoplates were coated with mAb 4G8 (epitope 17–24) (Covance) and blocked with 5% milk powder. Samples were added for 1 h. In separate plates, A $\beta$ <sub>40</sub> was detected with rabbit polyclonal PC-149 (Merck) and A $\beta$ <sub>42</sub> with a rabbit mAb BA3–9 (Covance), followed by biotinylated anti-rabbit IgG, extravidin alkaline phosphatase (Sigma) and 1 mg/ml 4-nitrophenol phosphate solution. Absorbance was measured at 405 nm and compared to a dose response of synthetic A $\beta$ <sub>1–40</sub>/A $\beta$ <sub>1–42</sub> (Bachem) suspended in the sample preparation buffer (as above) and sonicated.

## 2.14. PrP<sup>C</sup> filtration assays

Soluble PrP<sup>C</sup> was isolated from neuronal membrane preparations by a process including digestion with 0.2 units of phosphatidylinositol-phospholipase C (Sigma) followed by immunoaffinity and reverse phase chromatography on C18 columns as described (Bate and Williams, 2011). 1 nM soluble PrP<sup>C</sup> was incubated with A $\beta$  monomers or A $\beta$

oligomers for 1 h and then centrifuged through 50 kDa filters (Vivaspin - Sartorius) (Bate and Williams, 2011). The filtrate was collected and tested for the presence of PrP<sup>C</sup> by ELISA.

### 2.15. PrP<sup>C</sup> ELISA

The amount of PrP<sup>C</sup> in samples was determined by ELISA as described (Bate and Williams, 2011). Maxisorb immunoplates were coated with mAb ICSM18 (Dr M. Tayebi). Samples were added and PrP was detected with biotinylated mAb ICSM35 (Dr M. Tayebi) followed by extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenyl phosphate (Sigma). Absorbance was measured on a microplate reader at 405 nm and the amount of PrP in samples was calculated by reference to a standard curve of recombinant murine PrP (Prionics).

### 2.16. Preparation of Fab fragments

Fab (fraction antigen-binding) fragments were prepared from mAb 4F2 with the Immunopure Fab preparation Kit (Pierce) using immobilised papain using the manufacturer's instructions.

### 2.17. Statistical analysis

Comparison of treatment effects was carried out using Student's paired *t*-tests, one-way and two-way ANOVA with Bonferroni's post-hoc tests (IBM SPSS statistics 20). Error values reported are standard deviation (SD) and significance was determined where  $p < 0.01$ . Correlations between data sets were analysed using Pearson's bivariate coefficient (IBM SPSS statistics 20).

## 3. Results

### 3.1. A $\beta$ caused synapse damage in cultured neurons

Since the loss of synaptic proteins is a feature of AD that strongly correlates with cognitive decline (Counts et al., 2006; Masliah et al., 1991; Reddy et al., 2005) the effect of A $\beta$  on the amounts of synaptic proteins in cultured neurons was studied. A soluble brain extract from an AD patient contained forms of A $\beta$  including monomers, dimers and trimers (Fig. 1A). The addition of brain extract caused the loss of synaptophysin, CSP, synapsin-1 and vesicle-associated membrane protein (VAMP)-1, from neurons indicative of synapse degeneration (Fig. 1B). However, there was no loss of caveolin from treated neurons, nor was there any reduction in cell viability (98% survival  $\pm$  5 compared with 100% survival  $\pm$  6,  $n = 0$ ,  $p = 0.45$ ), indicating synapse degeneration without significant neuronal death. Immunodepletion with monoclonal antibody (mAb) 6E10 (reactive with A $\beta$ , amino acids 1–16) reduced the concentrations of both A $\beta_{40}$  (7.44 nM  $\pm$  0.8 SD to 0.5 nM  $\pm$  0.4 nM) and A $\beta_{42}$  (1.21 nM  $\pm$  0.16 to 0.16 nM  $\pm$  0.09 nM) in brain extracts, whereas mock-depletion had no significant effect on A $\beta_{40}$  (7.44 nM  $\pm$  0.8 to 7.12 nM  $\pm$  0.65 nM) or A $\beta_{42}$  (1.21 nM  $\pm$  0.22 to 1.09 nM  $\pm$  0.29 nM). The A $\beta$ -depleted brain extract still contained other peptides as shown by a coomassie blue stain (Supplementary Fig. 1). The addition of brain extracts to neurons resulted in the dose-dependent loss of synaptophysin (Fig. 1C) and CSP (Fig. 1D) (Bate and Williams, 2011). The addition of a brain extract that had been depleted of A $\beta$  did not affect the amounts of synaptophysin or CSP in neurons; observations consistent with the hypothesis that A $\beta$  is a major neurotoxin that causes synapse damage. A cautionary note that the 6E10 antibody used in these depletions reacts with an epitope common to multiple APP breakdown fragments including A $\beta_{40}$ , A $\beta_{42}$  and other carboxy-terminal fragments of APP (Hunter and Brayne, 2017).

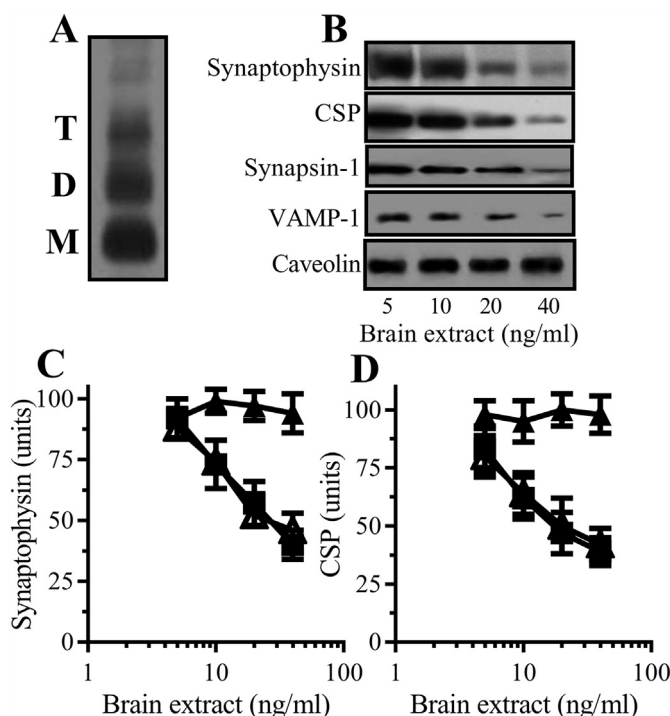


Fig. 1. Soluble brain extracts caused A $\beta$ -dependent synapse damage – (A) Western blot showing forms of A $\beta$  including monomers (M), dimers (D) and trimers (T) contained within a soluble brain extract. (B) Western blots showing the amounts of synaptophysin, CSP, synapsin-1, VAMP-1 and caveolin in cultured neurons that had been incubated with brain extract as shown. The amounts of synaptophysin (C) and CSP (D) neurons incubated with brain extract (■), A $\beta$ -depleted brain extract (▲) or mock-depleted brain extract (△) as shown for 24 h. Values are means  $\pm$  SD from triplicate experiments performed 4 times,  $n = 12$ . Some data points representing mock depleted brain extract (△) in (C) and (D) are not visible on the graphs as they are overlaid by data points representing the normal brain extract (■).

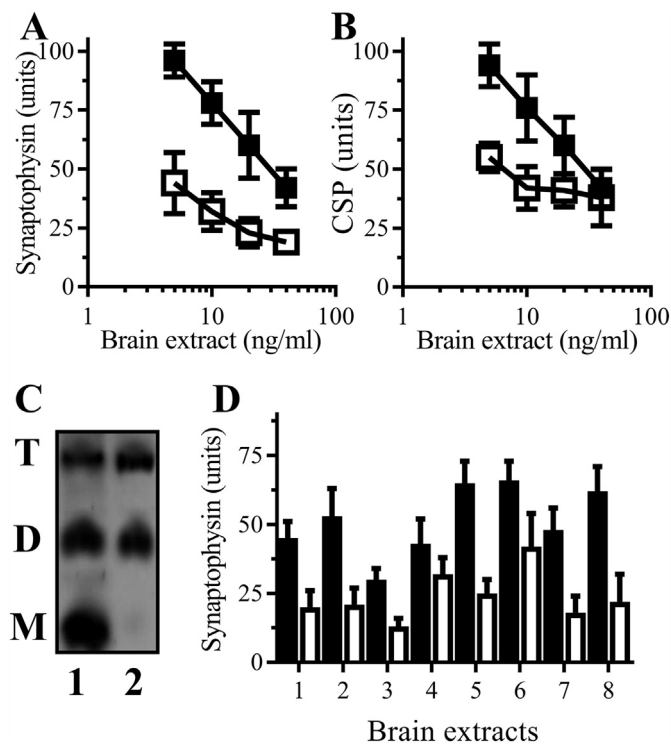
### 3.2. Removal of A $\beta$ monomers increased the brain extract-induced synapse damage

Brain extracts from AD patients contain a mixture of A $\beta$  monomers and soluble A $\beta$  oligomers, predominantly dimers and trimers (Klyubin et al., 2008; Mc Donald et al., 2010; Shankar et al., 2008). A $\beta$  oligomers were prepared by passage of the extract through a 10 kDa filter; the retained material contained A $\beta$  oligomers but lacked smaller APP fragments that we have called A $\beta$  monomers (Fig. 2C, lane 2). Brain extracts depleted of A $\beta$  monomers caused more synapse damage, as measured by the loss of synaptophysin (Fig. 2A) and CSP (Fig. 2B), when compared with the original brain extracts that contained A $\beta$  monomers (Fig. 2C, lane 1). To ensure that this observation was repeatable another 8 soluble brain extracts were depleted of monomers. In each sample the monomer-depleted extract caused significantly more synapse damage, as measured by the loss of synaptophysin, than did the untreated brain extracts (Fig. 2D). The concentrations of A $\beta_{40}$  and A $\beta_{42}$  varied between different samples. As there was a close correlation between A $\beta_{42}$  concentrations and synapse damage, whereas there was a poorer correlation between A $\beta_{40}$  and effect, we chose to quantify brain extracts on their A $\beta_{42}$  content. In the following experiments brain extracts were not pooled, rather monomers and oligomers were isolated from 3 main extracts and these were each measured in triplicate.

### 3.3. A $\beta$ monomers did not cause synapse damage

A $\beta$  monomers were prepared by passing brain extracts through a 10 kDa filter. This preparation contained only A $\beta$  monomers (Fig. 3A, lane 2) which eluted in a single peak from a Superdex 75 PC column (Fig. 3B). The addition of A $\beta$  monomer preparations containing 1 nM





**Fig. 2.** Removal of A $\beta$  monomers from brain extracts increases synapse damage – The amounts of synaptophysin (A) and CSP (B) in neurons incubated with soluble brain extract (■) or monomer-depleted brain extract (□) as shown. Values are means  $\pm$  SD from triplicate experiments performed 4 times,  $n = 12$ . (C) Immunoblot showing A $\beta$  monomers (M), dimers (D) and trimers (T) contained within brain extract (1) and brain extract retained by a 10 kDa filter (A $\beta$  oligomers) (2) separated by gel electrophoresis. (D) The amounts of synaptophysin in neurons incubated with 8 brain extracts (10 ng/ml) (■) or the same brain extracts that had been depleted of monomers (□). Values are means  $\pm$  SD from triplicate experiments performed twice,  $n = 6$ .

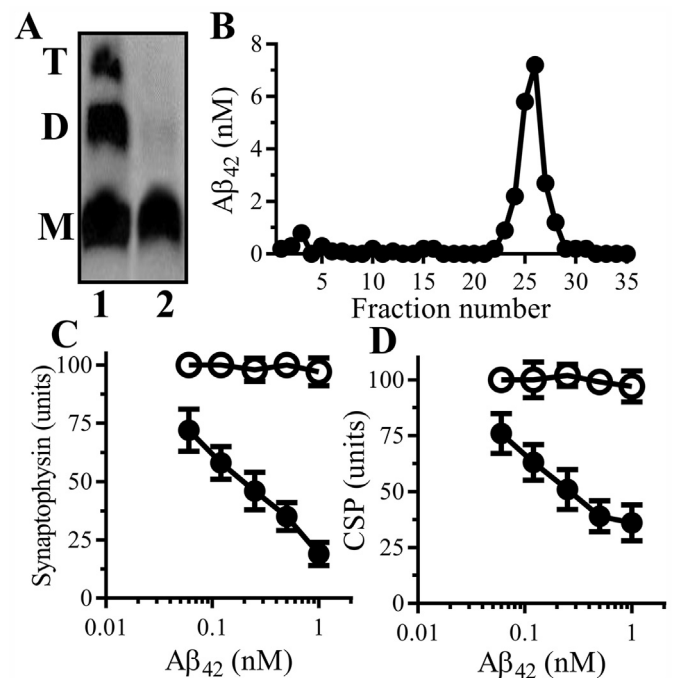
A $\beta_{42}$  did not affect the amounts of synaptophysin (Fig. 3C) or CSP (Fig. 3D) in neurons. In another experiment the addition of concentrated monomer preparations containing 20 nM A $\beta_{42}$  did not cause a significant reduction in the amounts of synaptophysin in neurons ( $98.4 \pm 3.5$  compared with  $100 \pm 3.8$ ,  $n = 12$ ,  $p = 0.34$ ). In contrast, A $\beta$  oligomers caused the loss of synaptophysin and CSP from neurons in a dose-dependent manner; there was a 50% reduction in synaptic proteins in neurons incubated with 0.2 nM A $\beta_{42}$  oligomers.

### 3.4. A $\beta$ oligomers but not monomers inhibit synaptic vesicle recycling

The uptake of fluorescent FM1-42 into recycling synaptic vesicles is commonly used to study synapse function (Klingauf et al., 1998). The addition of ionomycin for 1 min increased the amounts of FM1-43 taken up by synaptosomes in a dose-dependent manner (Fig. 4A). The addition of A $\beta$  inhibited the uptake of FM1-43 suggesting it inhibited synaptic vesicle recycling (Parodi et al., 2010). In the current study the ionomycin-induced uptake of FM1-43 into synaptosomes was reduced by incubation with brain extract (Fig. 4B). Immunodepletion studies showed that A $\beta$  was responsible for the inhibition of ionomycin-induced FM1-43 uptake by brain extract. The effects of brain extracts on the suppression of FM1-43 uptake were increased after the removal of monomers (Fig. 4C). A $\beta$  oligomers, but not A $\beta$  monomer preparations caused a dose-dependent inhibition of the ionomycin-induced uptake of FM1-43 (Fig. 4D).

### 3.5. A $\beta$ monomers inhibited A $\beta$ oligomer-induced synapse damage

The hypothesis that A $\beta$  monomers protected synapses was tested.

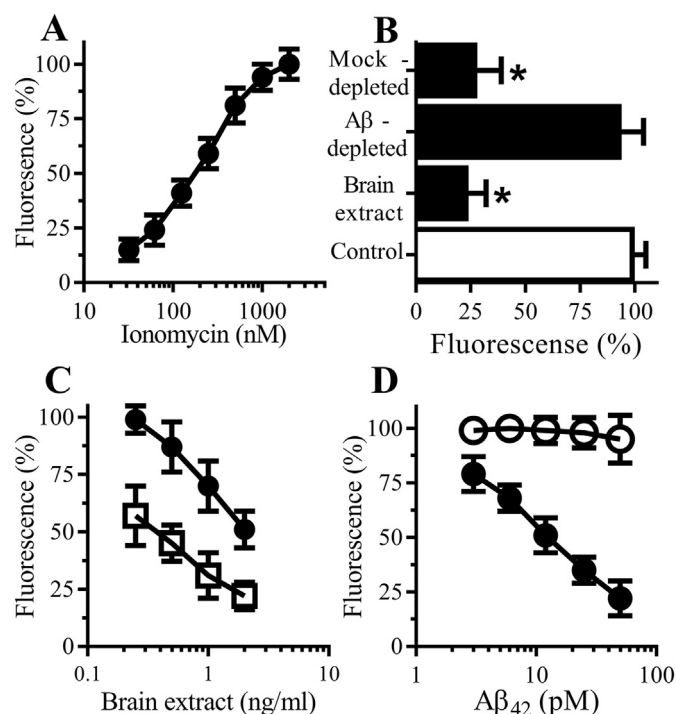


**Fig. 3.** A $\beta$  monomers do not cause synapse damage – (A) Immunoblot showing forms of A $\beta$  including monomers (M), dimers (D) and trimers (T) in soluble brain extract (1) and brain extract passed through a 10 kDa filter (A $\beta$  monomers) (2). (B) The concentrations of A $\beta$  in fractions eluted from a Superdex 75 PC column injected with a brain extract monomer preparation. Values are means of duplicates. The amounts of synaptophysin (C) and CSP (D) in neurons incubated with monomers (○) or oligomers (●) containing A $\beta_{42}$  as shown (0.0625, 0.125, 0.25, 0.5 and 1 nM). Values are means  $\pm$  SD from triplicate experiments performed 3 times,  $n = 9$ .

Pre-treatment with monomer preparations (containing 5 nM A $\beta_{42}$ , approximately 5 times the concentration of A $\beta$  oligomers that caused maximum synapse damage (1 nM)) protected neurons against the loss of synaptophysin (Fig. 5A) and CSP (Fig. 5B) triggered by A $\beta$  oligomers. The protective effect of monomers was stimulus specific; pre-treatment of neurons with monomers (containing 5 nM A $\beta_{42}$ ) did not alter the loss of synaptophysin from neurons incubated with phospholipase A $_2$ -activating peptide (PLAP), a peptide known to cause synapse damage (Bate et al., 2010) (Fig. 5C). Immunodepletion studies were used to examine the nature of the neuroprotective moiety in the monomer preparations. Immunodepletion with mAb 6E10 (reactive with A $\beta$ , amino acids 1–16) reduced the concentrations of A $\beta_{42}$  (from  $5 \text{ nM} \pm 0.2$  to  $0.1 \text{ nM} \pm 0.1 \text{ nM}$ ,  $n = 12$ ,  $p < 0.01$ ) whereas mock-depletion had no significant effect ( $5 \text{ nM} \pm 0.2$  compared with  $4.9 \text{ nM} \pm 0.3 \text{ nM}$ ,  $n = 12$ ,  $p = 0.27$ ). Neurons pre-treated with the A $\beta$ -depleted monomer preparations were not protected against synapse damage caused by A $\beta$  oligomers (containing 0.5 nM A $\beta_{42}$ ) (Fig. 5D). Pre-treatment with monomer preparations caused a dose-dependent inhibition of synapse damage when neurons were incubated with an oligomer preparation containing 0.5 nM A $\beta_{42}$  (Fig. 5E).

### 3.6. A $\beta$ monomers inhibited A $\beta$ oligomer-induced activation of cPLA $_2$ in synapses

Aberrant activation of cPLA $_2$  is thought to trigger synapse damage based on observations that A $\beta_{42}$  activates cPLA $_2$  (Anfuso et al., 2004; Shelat et al., 2008), that pharmacological inhibition of cPLA $_2$  protected cultured neurons against A $\beta$ -induced synapse damage (Bate et al., 2010) and ameliorated cognitive decline in a mouse model of AD (Sanchez-Mejia et al., 2008). For these reasons the effects of brain extracts upon cPLA $_2$  in synapses were examined. The addition of brain extracts containing 1 nM A $\beta_{42}$  increased the amounts of activated

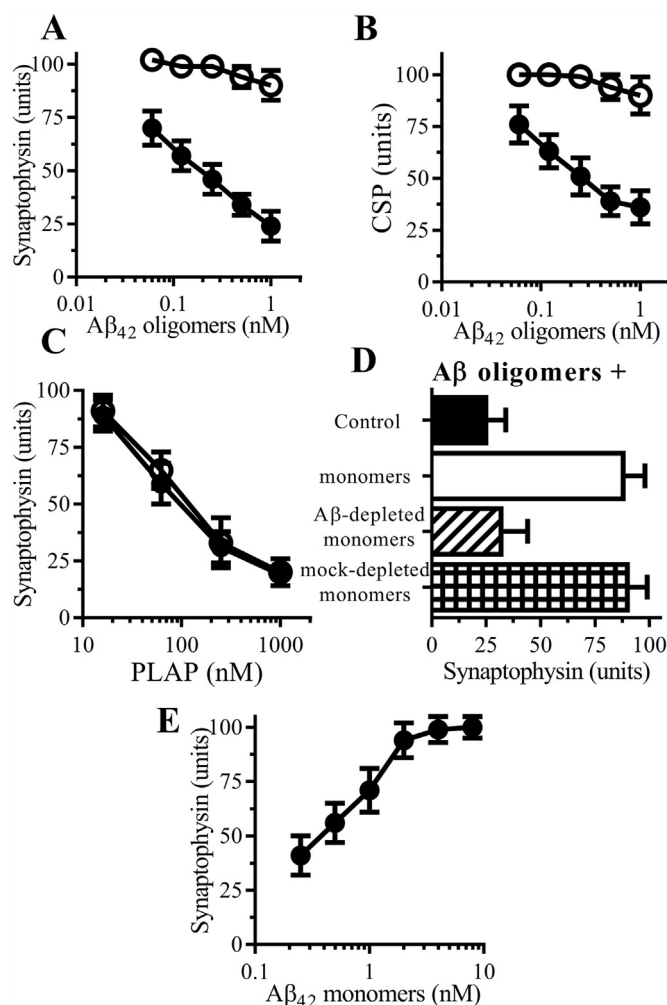


**Fig. 4.** A $\beta$  oligomers inhibit synaptic vesicle recycling – (A) The amounts of FM1-43 in synaptosomes incubated with ionomycin as shown (●). Values are mean % fluorescence (100% = maximum fluorescence)  $\pm$  SD from triplicate experiments performed 3 times (n = 9). (B) The amounts of FM1-43 in synaptosomes incubated with control medium (□), brain extract, A $\beta$ -depleted brain extract or mock-depleted brain extract (■) for 30 min and incubated with 1  $\mu$ M ionomycin. Values are means  $\pm$  SD from triplicate experiments performed 3 times (n = 9). \* = fluorescence significantly less than controls. (C) The amounts of FM1-43 in synaptosomes incubated with brain extract (●) or monomer-depleted brain extract (□) for 30 min and incubated with 1  $\mu$ M ionomycin. Values are means  $\pm$  SD from triplicate experiments performed 3 times (n = 9). (D) The amounts of FM1-43 in synaptosomes incubated with oligomer (●) or monomer (○) preparations containing A $\beta$ <sub>42</sub> for 30 min as shown and incubated with 1  $\mu$ M ionomycin. Values are means  $\pm$  SD from triplicate experiments performed 3 times (n = 9).

cPLA<sub>2</sub> found in synaptosomes (Fig. 6A) whereas brain extracts depleted of A $\beta$  had a reduced effect. A $\beta$  oligomers, but not A $\beta$  monomers, activated synaptic cPLA<sub>2</sub> (Fig. 6B). Pre-treatment of synaptosomes with monomer preparations (containing 1 nM A $\beta$ <sub>42</sub>, approximately 5 times the concentration of A $\beta$  oligomers that caused maximum activation of cPLA<sub>2</sub> (0.2 nM)) reduced the activation of cPLA<sub>2</sub> by A $\beta$  oligomers (Fig. 6C) but not by PLAP (Fig. 6D) indicating that monomers did not have a direct effect upon this enzyme. Immunodepletion studies demonstrated that it was A $\beta$  within the monomer preparations that was responsible for blocking the A $\beta$  oligomer-induced activation of synaptic cPLA<sub>2</sub> (Fig. 6E) and the production of prostaglandin (PG)E<sub>2</sub> (Fig. 6F), a bioactive lipid found in high concentrations the brains of AD patients (Montine et al., 1999) which causes synapse degeneration in cultured neurons (Bate et al., 2010). Collectively these results suggest that it is A $\beta$  monomers that inhibit the A $\beta$  oligomer-induced activation of synaptic cPLA<sub>2</sub> and PGE<sub>2</sub> production.

### 3.7. A $\beta$ monomers inhibited the A $\beta$ oligomer-induced increase in cholesterol concentrations

The concentration of cholesterol in cell membranes is a critical factor involved in neurodegeneration (Maxfield and Tabas, 2005). The addition of brain extracts significantly increased the concentrations of cholesterol in synaptosomes (West et al., 2017) (Fig. 7A) an observation that is consistent with reports of increased cholesterol in A $\beta$  positive synapses in the cortex of Alzheimer's patients (Gyls et al., 2007). This effect of brain extracts was lost after the removal of A $\beta$ . Further studies

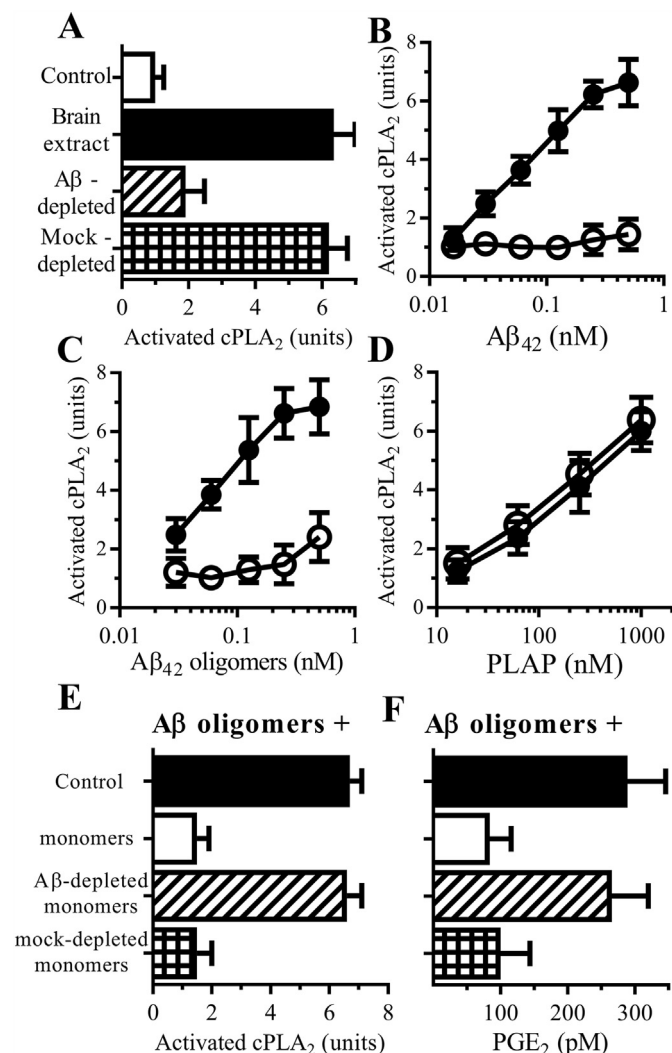


**Fig. 5.** A $\beta$  monomers inhibit A $\beta$  oligomer-induced synapse damage - The amounts of synaptophysin (A) and CSP (B) in neurons pre-treated with control medium (●) or monomers (containing 5 nM A $\beta$ <sub>42</sub>) (○) and incubated with A $\beta$  oligomers as shown. Values are means  $\pm$  SD from triplicate experiments performed 3 times, n = 9. (C) The amounts of synaptophysin in neurons pre-treated with control medium (●) or monomer preparations containing 5 nM A $\beta$ <sub>42</sub> (○) and incubated with PLAP. Values are means  $\pm$  SD from triplicate experiments performed 3 times, n = 9. (D) The amounts of synaptophysin in neurons pre-treated with control medium (■), monomers (□), A $\beta$ -depleted monomers (striped bar) or mock-depleted monomers (hatched bar) and incubated with an oligomer preparation containing 0.5 nM A $\beta$ <sub>42</sub>. Values are means  $\pm$  SD from triplicate experiments performed 3 times, n = 9. (E) The amounts of synaptophysin in neurons pre-treated with monomers containing A $\beta$ <sub>42</sub> as shown (●) and incubated with an oligomer preparation containing 0.5 nM A $\beta$ <sub>42</sub>. Values are means  $\pm$  SD from triplicate experiments performed 2 times, n = 6.

showed that A $\beta$  oligomers increased synaptic cholesterol while monomers did not (Fig. 7B). When incubated with brain extracts containing between 1 and 0.06 nM A $\beta$ <sub>42</sub> there were significant correlations between concentrations of cholesterol and activated cPLA<sub>2</sub> (Fig. 7C) and PGE<sub>2</sub> (Fig. 7D). Next, the effects of A $\beta$  monomers on the A $\beta$  oligomer-induced increase in synaptic cholesterol concentrations were studied. Monomer preparations (containing 5 nM A $\beta$ <sub>42</sub>) blocked the increase in cholesterol concentrations caused by A $\beta$  oligomers (containing 0.5 nM A $\beta$ <sub>42</sub>) (Fig. 7E). Immunodepletion studies showed that A $\beta$  monomers were responsible for blocking the A $\beta$  oligomer-induced increase in cholesterol.

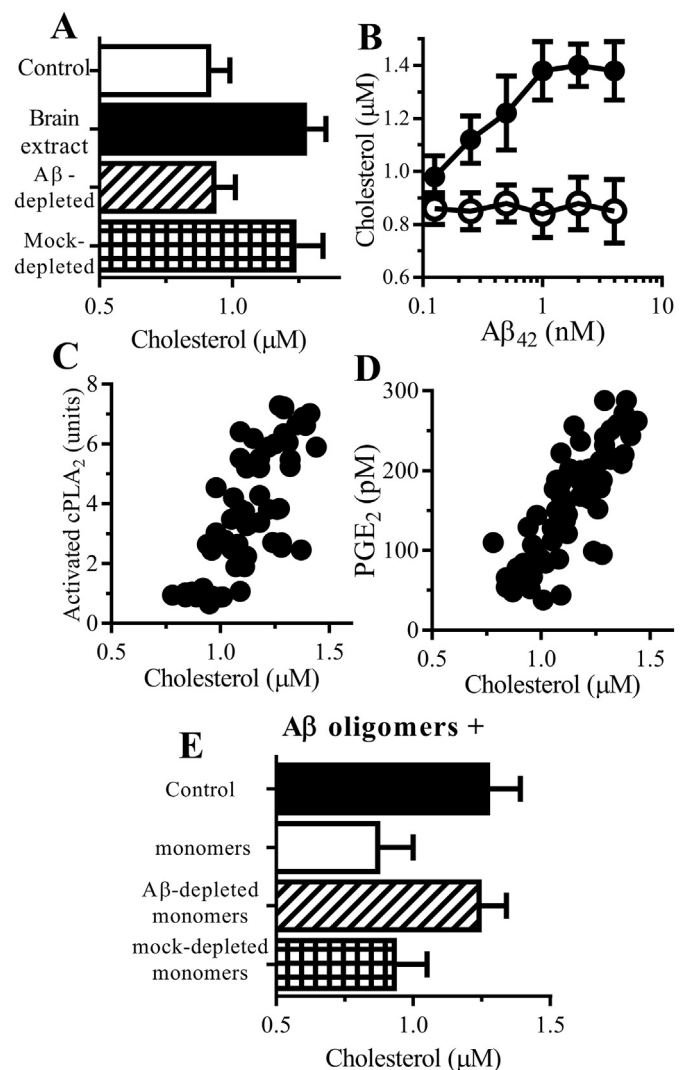
### 3.8. A $\beta$ monomers inhibited A $\beta$ oligomer-induced aggregation of PrP<sup>C</sup>

The observations that cross-linkage of PrP<sup>C</sup> by antibodies leads to neurodegeneration (Solforosi et al., 2004) and that A $\beta$  oligomers cause



**Fig. 6.** Aβ monomers inhibit the Aβ oligomer-induced activation of synaptic cPLA<sub>2</sub> - (A) The amounts of activated cPLA<sub>2</sub> in synaptosomes incubated with control medium (□) brain extract (■), Aβ-depleted brain extract (striped bar) or mock-depleted brain extract (hatched bar). Values are means ± SD from triplicate experiments performed 3 times, n = 9. (B) The amounts of activated cPLA<sub>2</sub> in synaptosomes incubated with oligomers (●) or monomers (○) containing Aβ<sub>42</sub> as shown. Values are means ± SD from triplicate experiments performed 3 times, n = 9. (C) The amounts of activated cPLA<sub>2</sub> in synaptosomes pre-treated with control medium (●) or monomers containing 1 nM Aβ<sub>42</sub> (○) and incubated with oligomers containing Aβ<sub>42</sub> (C) or PLAP (D) as shown. Values are means ± SD from triplicate experiments performed 3 times, n = 9. (E) The amounts of activated cPLA<sub>2</sub> (E) and PGE<sub>2</sub> (F) in synaptosomes pre-treated with control medium (■), monomers containing 1 nM Aβ<sub>42</sub> (□), Aβ-depleted monomers (striped bars) or mock-depleted monomers (hatched bars) and incubated with oligomers containing 0.5 nM Aβ<sub>42</sub>. Values are means ± SD from triplicate experiments performed 3 times, n = 9.

PrP<sup>C</sup> aggregation (Bate and Williams, 2011) suggested that aggregation of PrP<sup>C</sup> is a critical factor that initiates synapse damage and the pathogenesis of AD. Here we show that PrP<sup>C</sup> incubated with brain extract formed complexes containing at least two PrP<sup>C</sup> molecules as shown by immunoblot (Fig. 8A). This qualitative study was supported by quantitative studies in which 1 nM soluble PrP<sup>C</sup> was passed through a 50 kDa filter as described (Bate and Williams, 2011). Although PrP<sup>C</sup> has a molecular weight that is dependent upon glycosylation it does not exceed 37 kDa and consequently all soluble PrP<sup>C</sup> passed through a 50 kDa filter. PrP<sup>C</sup> incubated with Aβ monomers (1 nM Aβ<sub>42</sub>) also passed through a 50 kDa filter whereas incubation with Aβ oligomers (1 nM Aβ<sub>42</sub>) reduced the amounts of PrP<sup>C</sup> that passed through a 50 kDa filter by approximately 80%. The formation of PrP<sup>C</sup> complexes of > 50 kDa was lost after the removal of Aβ from brain extracts (Fig. 8B)

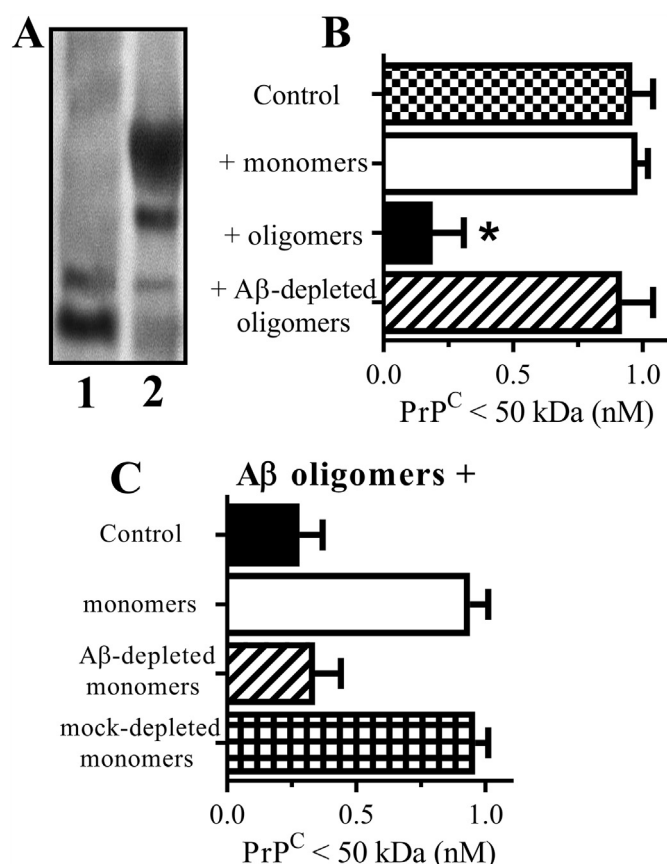


**Fig. 7.** Aβ monomers inhibit the Aβ oligomer-induced increase in synaptic cholesterol - (A) The concentrations of cholesterol in synaptosomes incubated with control medium (□) brain extract (■), Aβ-depleted brain extract (striped bar) or mock-depleted brain extract (hatched bar). Values are means ± SD from triplicate experiments performed 3 times, n = 9. (B) The concentrations of cholesterol in synaptosomes incubated with oligomers (●) or monomers (○) containing Aβ<sub>42</sub> as shown. Values are means ± SD from triplicate experiments performed 3 times, n = 9. (C) The concentrations of cholesterol and the amounts of activated cPLA<sub>2</sub> (C), Pearson's coefficient = 0.75, p < 0.01 and the concentrations of PGE<sub>2</sub> produced (D) Pearson's coefficient = 0.79, p < 0.01. (E) The concentrations of cholesterol in synaptosomes pre-treated with control medium (■), monomers (□), Aβ-depleted monomers (striped bar) or mock-depleted monomers (hatched bar) and incubated with oligomers containing 0.5 nM Aβ<sub>42</sub>. Values are means ± SD from triplicate experiments performed 3 times (n = 9).

indicating that Aβ oligomers cross-link PrP<sup>C</sup>. To test the hypothesis that the binding of Aβ monomers to PrP<sup>C</sup> block the aggregation of PrP<sup>C</sup> by Aβ oligomers, soluble PrP<sup>C</sup> was pre-treated with monomers (containing 1 nM Aβ<sub>42</sub>) and incubated with oligomers (containing 1 nM Aβ<sub>42</sub>). Pre-treatment of soluble PrP<sup>C</sup> with Aβ monomers significantly reduced the Aβ oligomer-induced formation of PrP<sup>C</sup> complexes > 50 kDa (Fig. 8C).

### 3.9. Fab fragments block the mAb-mediated cross-linkage of PrP<sup>C</sup> and synapse damage

The cross-linkage of PrP<sup>C</sup> by mAbs caused neurodegeneration in vivo (Solfrosi et al., 2004) and synapse damage in cultured neurons (Bate and Williams, 2011). The PrP<sup>C</sup>-reactive mAb 4F2 mimicked some of the effects of Aβ oligomers on synaptosomes, including increasing



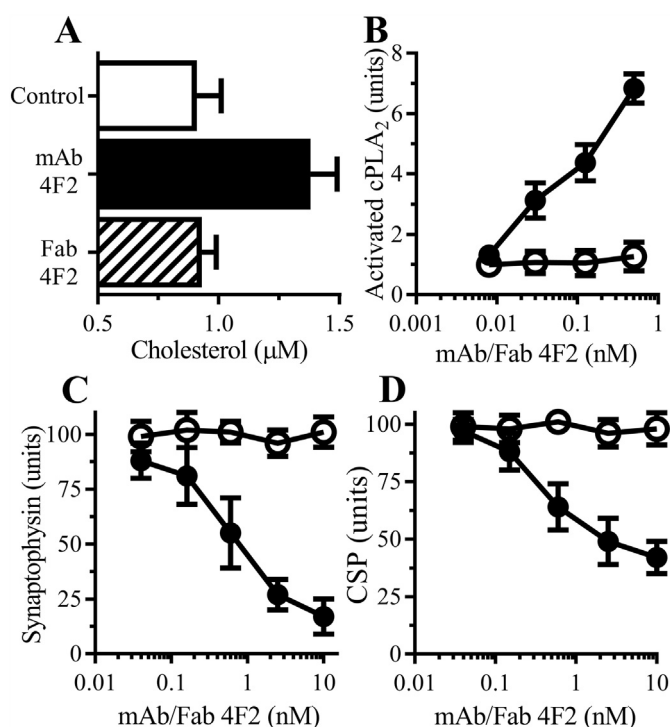
**Fig. 8.** Aβ oligomers cause aggregation of PrP<sup>C</sup> – (A) Immunoblot showing PrP<sup>C</sup> in preparations of (1) PrP<sup>C</sup> or (2) PrP<sup>C</sup> mixed with Aβ oligomers separated by non-denaturing gel electrophoresis. (B) The concentrations of PrP<sup>C</sup> passing through a 50 kDa filter after incubation with control medium (checkerboard bar), monomers (□), oligomers (■) or Aβ-depleted oligomers (striped bars). Values are means from triplicate experiments performed 4 times, *n* = 12. \* = PrP<sup>C</sup> < 50 kDa significantly less than in controls. (C) The concentrations of PrP<sup>C</sup> passing through a 50 kDa filter after PrP<sup>C</sup> was incubated with Aβ oligomers mixed with control medium (■), monomers (□), Aβ-depleted monomers (striped bar) or mock-depleted monomers (hatched bars). Values are means ± SD from triplicate experiments performed 3 times, *n* = 9.

cholesterol concentrations (Fig. 9A) and activating cPLA<sub>2</sub> (Fig. 9B). These effects were not seen in synaptosomes incubated with monovalent Fab fragments derived from 4F2 that do not cross-link PrP<sup>C</sup>. When the mAb/Fab fragments were incubated with cultured neurons, the divalent mAb 4F2 caused a dose-dependent loss of synaptophysin (Fig. 9C) and CSP (Fig. 9D) which was not seen with the monovalent Fab fragments. Pre-treatment of synaptosomes with Fab fragments from 4F2 (but not Fab fragments prepared from a control mouse IgG mAb) significantly reduced the mAb 4F2-induced increase in cholesterol concentrations (Fig. 10A) and the mAb 4F2-induced activation of cPLA<sub>2</sub> (Fig. 10B). Pre-treatment of neurons with 4F2 Fab fragments, but not Fab fragments from a control IgG, also reduced the mAb 4F2-induced reduction of synaptophysin (Fig. 10C) and CSP (Fig. 10D) from neurons.

#### 4. Discussion

The principal finding of this study is that Aβ monomer preparations reduced the Aβ oligomer-induced synapse damage. This protection was associated with Aβ monomers reducing the Aβ oligomer-induced aggregation of PrP<sup>C</sup> and the subsequent increase in synaptic cholesterol concentrations and the activation of cPLA<sub>2</sub>.

Soluble brain extracts were used in this study since they contain a mixture of monomers and low-n oligomers, that are similar in size, solubility, stability and potency to the Aβ previously reported in the

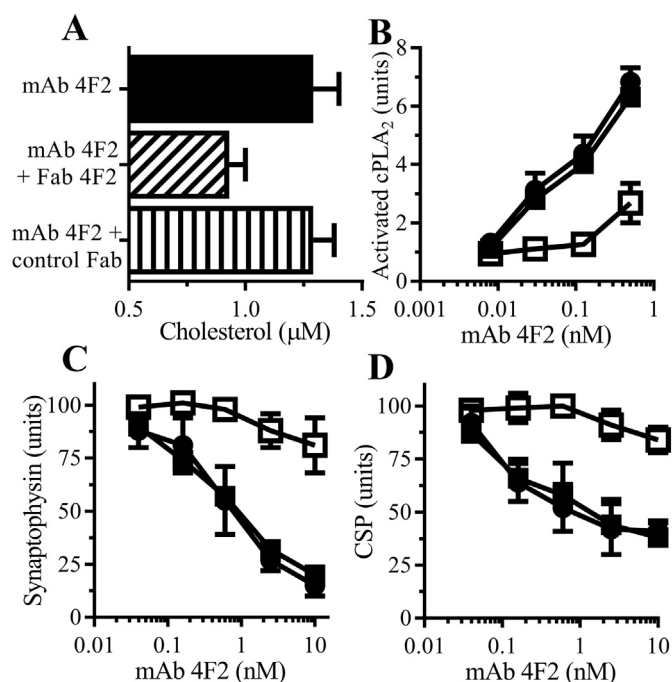


**Fig. 9.** A PrP<sup>C</sup> mAb increases synaptic cholesterol, activates synaptic cPLA<sub>2</sub> and triggers synapse damage – (A) The concentrations of cholesterol in synaptosomes treated with control medium (□), 1 nM mAb 4F2 (■) or 2 nM 4F2 Fab fragments (striped bar). Values are means ± SD from triplicate experiments performed 3 times (*n* = 9). \* = cholesterol significantly higher than in control synaptosomes. (B) The amounts of activated cPLA<sub>2</sub> in synaptosomes treated with mAb 4F2 (●) or 4F2 Fab fragments (○) as shown. Values are means ± SD from triplicate experiments performed 3 times, *n* = 9. (C) The amounts of synaptophysin in neurons treated with mAb 4F2 (●) or 4F2 Fab fragments (○). Values are means ± SD from triplicate experiments performed 3 times, *n* = 9. (D) The amounts of CSP in neurons treated with mAb 4F2 (●) or 4F2 Fab fragments (○). Values are means ± SD from triplicate experiments performed 3 times, *n* = 9.

CSF from AD patients (Bibl et al., 2007; McLean et al., 1999; Mehta et al., 2000). Thus, the presence of brain extracts containing picomolar concentrations of Aβ<sub>42</sub> caused synapse damage to cultured neurons. The initial observations, that the removal of Aβ monomers from brain extracts increased synapse damage, suggested that Aβ monomers have a protective role. This hypothesis was supported by experiments using Aβ monomers collected by filtration. At the maximum concentration of monomers used (containing 20 nM Aβ<sub>42</sub>) they did not cause synapse damage. In contrast, the oligomer preparation that reduced the synaptophysin content of neurons by 50% contained approximately 0.2 nM Aβ<sub>42</sub> (Fig. 3C). We note that in addition to Aβ<sub>40</sub> and Aβ<sub>42</sub> the preparations used in this study are likely to contain other APP fragments which may contribute to the activity reported and consequently the word “monomers” was used to include all Aβ fragments passing through a 10 kDa filter. The activity of monomer preparations was removed by immunodepletion with mAb 6E10, indicating that the bioactive peptides contained a specific epitope. However the methods used in this study do not differentiate whether those peptides were Aβ<sub>40</sub>, Aβ<sub>42</sub> or other APP fragments sharing this epitope.

Recent studies suggest that Aβ caused synapse damage as a consequence of aberrant cell signalling. As synthetic Aβ<sub>42</sub> monomers activate the phosphatidylinositol-3-kinase pathway (Giuffrida et al., 2009) and insulin signalling (Giuffrida et al., 2012) it is possible that Aβ monomers and Aβ oligomers activate different signalling pathways. Here we show that the Aβ oligomers, but not Aβ monomers, activate cPLA<sub>2</sub> in synaptosomes, consistent with reports that pharmacological inhibition of cPLA<sub>2</sub> protected cultured neurons against Aβ-induced synapse damage (Bate et al., 2010) and ameliorated cognitive decline in a





**Fig. 10.** Fab 4F2 fragments protect neurons against mAb 4F2-induced synapse damage – (A) The concentrations of cholesterol in synaptosomes incubated with 2 nM mAb 4F2 (■), or mixtures containing 2 nM 4F2 Fab fragments and 2 nM mAb 4F2 (striped bar) or 2 nM control Fab fragments and 2 nM mAb 4F2 (vertical striped bar). Values are means  $\pm$  SD from triplicate experiments performed 3 times ( $n = 9$ ). \* = cholesterol significantly lower than in synaptosomes incubated with mAb 4F2. (B) The amounts of activated cPLA<sub>2</sub> in synaptosomes pre-treated with control medium (●), 2 nM 4F2 Fab fragments (□) or 2 nM control IgG Fab fragments (■) and incubated with mAb 4F2. Values are means  $\pm$  SD from triplicate experiments performed 3 times,  $n = 9$ . The amounts of synaptophysin (C) and CSP (D) in neurons pre-treated with control medium (●), 2 nM 4F2 Fab fragments (□) or 2 nM control Fab fragments (■) and incubated with mAb 4F2. Values are means  $\pm$  SD from triplicate experiments performed 3 times,  $n = 9$ .

mouse model of AD (Sanchez-Mejia et al., 2008). The activation of cPLA<sub>2</sub> by A $\beta$  involves the translocation of cPLA<sub>2</sub> to lipid rafts and is sensitive to cholesterol depletion (Bate and Williams, 2011). Here we demonstrate that A $\beta$  oligomers, but not A $\beta$  monomers, increased cholesterol concentrations and activated cPLA<sub>2</sub> within synapses.

PrP<sup>C</sup> that is highly expressed at synapses (Brown, 2001; Herms et al., 1999) has been identified as a receptor for A $\beta$ <sub>42</sub> (Balducci et al., 2010; Lauren et al., 2009) and the activation of cPLA<sub>2</sub> in synapses by A $\beta$  involves PrP<sup>C</sup> (Bate and Williams, 2011). PrP<sup>C</sup> is attached to membranes via a glycosylphosphatidylinositol (GPI) anchor (Stahl et al., 1987) and the cross-linkage of GPI-anchored proteins leads to membrane realignment and cell activation (Chen et al., 2006; Suzuki et al., 2007). More specifically, the antibody-mediated cross-linkage of PrP<sup>C</sup> promotes the formation of lipid rafts and cell signalling in T cells (Stuermer et al., 2004). Raft formation is associated with the oligomerization of proteins (Engelman, 2005), specifically GPI-anchored proteins (Egeling et al., 2009; Suzuki et al., 2012), as the aggregation of membrane components leads to the reorganization of cell membranes (Hammond et al., 2005; Lingwood et al., 2008). We hypothesise that it is the aggregation of PrP<sup>C</sup> caused by A $\beta$  oligomers that is an essential process leading to synapse damage. Activation of cell signalling pathways is associated with the aggregation of proteins (Suzuki et al., 2007) and here we show that A $\beta$  oligomers, but not monomers, cause PrP<sup>C</sup> to aggregate, increase synaptic cholesterol concentrations and activate cPLA<sub>2</sub>. This hypothesis is supported by our observations that the cross-linkage of PrP<sup>C</sup> by mAb 4F2 mimicked some of the effects of A $\beta$  oligomers upon synaptosomes, including increasing cholesterol concentrations and activating cPLA<sub>2</sub>; mAb 4F2 also caused synapse degeneration in cultured neurons. In contrast, no changes in synaptic

cholesterol concentrations, activation of cPLA<sub>2</sub> or synapse damage were observed in synaptosomes or neurons incubated with monovalent Fab fragments derived from mAb 4F2. The reorganization of rafts in the outer leaflet, in response to stimuli, results in the sorting of intracellular signalling molecules on the inner, cytoplasmic leaflet (Eisenberg et al., 2006; Hunter, 2000). Thus, the clustering of individual rafts exposes proteins to new membrane environments and can initiate signalling. Consequently the aggregation of cell surface PrP<sup>C</sup> can cause the clustering of receptor-associated signalling molecules including cPLA<sub>2</sub> within lipid raft platforms. It should be noted that A $\beta$  has been reported to bind to numerous other proteins (Jarosz-Griffiths et al., 2016) meaning that A $\beta$  oligomers have the potential to link PrP<sup>C</sup> with other proteins. The heterogeneity of complexes formed between A $\beta$  oligomers and numerous different “receptors” may help to explain how A $\beta$  causes the wide variety of cellular responses reported.

We report that A $\beta$  monomer preparations protected neurons against the A $\beta$  oligomer-induced synapse damage. These monomers also reduced the A $\beta$  oligomer-induced increase in synaptic cholesterol concentrations and activation of cPLA<sub>2</sub> that is closely associated with synapse damage. A $\beta$  monomers did not affect the activation of cPLA<sub>2</sub> by PLAP, nor did they affect PLAP-induced synapse damage, indicating that they interfere with a stimulus-specific pathway. A $\beta$  monomers did not cause aggregation of PrP<sup>C</sup>, consistent with the hypothesis that A $\beta$  contains a single PrP<sup>C</sup>-binding site and consequently A $\beta$  oligomers, but not A $\beta$  monomers cause the aggregation of PrP<sup>C</sup>. The presence of A $\beta$  monomers blocks the A $\beta$  oligomer-induced aggregation of PrP<sup>C</sup> which has been shown to cause cell signalling and synapse degeneration (Bate and Williams, 2012). These results contrast studies that showed that synthetic A $\beta$ <sub>42</sub> monomers did not bind to recombinant PrP23–231 (Freir et al., 2011; Nicoll et al., 2013; Um et al., 2012) and suggest that the difference may be the sources of A $\beta$  and PrP proteins used in binding assays (synthetic vs natural). This study also used natural rather than recombinant PrP proteins. The post-translational modifications of PrP include a GPI anchor and N-linked glycosylation, factors which can affect structure and function of some proteins (Buttkofer et al., 2001; Elfrink et al., 2008; Kemble et al., 1993). For example, native PrP<sup>C</sup> contains sialic acid (Rudd et al., 2001; Stahl et al., 1992) a glycan implicated in A $\beta$  binding and structure (Hong et al., 2014; Kakio et al., 2002).

Critically we found that pre-treatment with A $\beta$  monomer preparations prevented the A $\beta$  oligomer-induced aggregation of PrP<sup>C</sup>. These preparations contain a mixture of A $\beta$  species and consequently it was not possible to identify the precise form of A $\beta$  that is responsible for this effect. It is possible that more than one A $\beta$  species is involved. We hypothesise that A $\beta$  monomers compete with A $\beta$  oligomers for binding sites on PrP<sup>C</sup>. Thus, the binding of A $\beta$  monomers to PrP<sup>C</sup> prevented the binding of A $\beta$  oligomers and subsequent cross-linkage of PrP<sup>C</sup>. This hypothesis is supported by studies using Fab fragments of the PrP<sup>C</sup>-binding mAb 4F2. Pre-treatment of synaptosomes with monovalent Fab fragments of 4F2 (that do not cross-link PrP<sup>C</sup>) significantly reduced both mAb 4F2-induced increase in cholesterol concentrations and mAb 4F2-induced activation of cPLA<sub>2</sub>. Furthermore, pre-treatment of neurons with 4F2 Fab fragments blocked the mAb 4F2-induced synapse damage.

Factors thought to be involved in determining synapse damage in AD include the length of the A $\beta$  fragment, oligomer size and the conformation of A $\beta$ . Although many studies measure A $\beta$  levels as a marker of disease progression, they often do not differentiate between A $\beta$  monomers and oligomers. These results suggest that the concentration of A $\beta$  monomers is another factor that should be considered in determining disease progression. Thus, the presence of high concentrations of A $\beta$  monomers could protect neurons against synapse damage even in the presence of high concentrations of A $\beta$  oligomers. However, the role of A $\beta$  monomers is not completely clear as others have reported that the presence of A $\beta$  monomers was associated with dementia (McDonald et al., 2010). It should be noted that this work was carried out

using human brain-derived A $\beta$  preparations incubated with mouse synaptosomes and neurons. Practicalities prevented the ideal situation which would have been to use human neurons to reduce variation occurring from cross species experiments.

## 5. Conclusions

In conclusion these results support the hypothesis that A $\beta$  monomers have a protective role by reducing synapse damage caused by A $\beta$  oligomers. They are consistent with the hypothesis that A $\beta$  monomers compete with A $\beta$  oligomers for binding sites on PrP<sup>C</sup> and that the binding of A $\beta$  monomers prevents the A $\beta$  oligomer-induced aggregation of PrP<sup>C</sup> that triggers aberrant activation of cPLA<sub>2</sub> and synapse damage. These observations suggest that AD may develop as a result of changes in the A $\beta$  monomer:oligomer ratio.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nbd.2017.12.007>.

## Competing interest

The authors declare that there are no competing interests.

## Authors' contributions

CB: conception and design, data collection and analysis, manuscript writing and revision. AW: conception and design and manuscript writing. Both authors read and approved the final manuscript.

## Acknowledgements

This work was supported by the European Commission FP6 “Neuroprion” – Network of Excellence. We also thank Professor J. Grassi for mAb 4F2 and Dr. M. Tayebi for mAbs ICSM18 and ICSM35.

## References

- Anfuso, C.D., et al., 2004. Amyloid  $\beta$ (1–42) and its  $\beta$ (25–35) fragment induce activation and membrane translocation of cytosolic phospholipase A<sub>2</sub> in bovine retina capillary pericytes. *Biochim. Biophys. Acta* 1686, 125–138.
- Balducci, C., et al., 2010. Synthetic amyloid- $\beta$  oligomers impair long-term memory independently of cellular prion protein. *Proc. Natl. Acad. Sci. U. S. A.* 107, 2295–2300.
- Bate, C., Williams, A., 2011. Amyloid- $\beta$ -induced synapse damage is mediated via cross-linkage of the cellular prion protein. *J. Biol. Chem.* 286, 37955–37963.
- Bate, C., Williams, A., 2012. Neurodegeneration induced by the clustering of sialylated glycosylphosphatidylinositols of prion proteins. *J. Biol. Chem.* 287, 7935–7944.
- Bate, C., et al., 2010. Phospholipase A<sub>2</sub> inhibitors protect against prion and A $\beta$  mediated synapse degeneration. *Mol. Neurodegener.* 5, 13.
- Bibl, M., et al., 2007. Validation of amyloid- $\beta$  peptides in CSF diagnosis of neurodegenerative dementias. *Mol. Psychiatry* 12, 671–680.
- Brinkmalm, G., et al., 2012. An online nano-LC-ESI-FTICR-MS method for comprehensive characterization of endogenous fragments from amyloid beta and amyloid precursor protein in human and cat cerebrospinal fluid. *J. Mass Spectrom.* 47, 591–603.
- Brown, D.R., 2001. Prion and prejudice: normal protein and the synapse. *Trends Neurosci.* 24, 85–90.
- Butikofer, P., et al., 2001. GPI-anchored proteins: now you see 'em, now you don't. *FASEB J.* 15, 545–548.
- Chen, Y., et al., 2006. Transient anchorage of cross-linked glycosyl-phosphatidylinositol-anchored proteins depends on cholesterol, Src family kinases, caveolin, and phosphoinositides. *J. Cell Biol.* 175, 169–178.
- Counts, S.E., et al., 2006. Differential expression of synaptic proteins in the frontal and temporal cortex of elderly subjects with mild cognitive impairment. *J. Neuropathol. Exp. Neurol.* 65, 592–601.
- De Strooper, B., et al., 2010. The secretases: enzymes with therapeutic potential in Alzheimer disease. *Nat. Rev. Neurol.* 6, 99–107.
- Dunkley, P.R., et al., 2008. A rapid Percoll gradient procedure for preparation of synaptosomes. *Nat. Protoc.* 3, 1718–1728.
- Eggeling, C., et al., 2009. Direct observation of the nanoscale dynamics of membrane lipids in a living cell. *Nature* 457, 1159–1162.
- Eisenberg, S., et al., 2006. Clustering of raft-associated proteins in the external membrane leaflet modulates internal leaflet H-Ras diffusion and signaling. *Mol. Cell Biol.* 26, 7190–7200.
- Elfrink, K., et al., 2008. Structural changes of membrane-anchored native PrP<sup>C</sup>. *Proc. Natl. Acad. Sci. U. S. A.* 105 (31), 10815–10819.
- Engelman, D.M., 2005. Membranes are more mosaic than fluid. *Nature* 438, 578–580.
- Freir, D.B., et al., 2011. Interaction between prion protein and toxic amyloid beta assemblies can be therapeutically targeted at multiple sites. *Nat. Commun.* 2, 336.
- Giuffrida, M.L., et al., 2009.  $\beta$ -Amyloid monomers are neuroprotective. *J. Neurosci.* 29, 10582–10587.
- Giuffrida, M., et al., 2012. Beta-amyloid monomer and insulin/IGF-1 signaling in Alzheimer's disease. *Mol. Neurobiol.* 46, 605–613.
- Gylys, K.H., et al., 2007. Increased cholesterol in A[beta]-positive nerve terminals from Alzheimer's disease cortex. *Neurobiol. Aging* 28, 8–17.
- Hammond, A.T., et al., 2005. Crosslinking a lipid raft component triggers liquid ordered-liquid disordered phase separation in model plasma membranes. *Proc. Natl. Acad. Sci. U. S. A.* 102, 6320–6325.
- Hardy, J., Selkoe, D.J., 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297, 353–356.
- Hermes, J., et al., 1999. Evidence of presynaptic location and function of the prion protein. *J. Neurosci.* 19, 8866–8875.
- Hong, S., et al., 2014. Soluble Abeta oligomers are rapidly sequestered from brain ISF in vivo and bind GM1 ganglioside on cellular membranes. *Neuron* 82, 308–319.
- Hunter, T., 2000. Signaling—2000 and beyond. *Cell* 100, 113–127.
- Hunter, S., Brayne, C., 2017. Do anti-amyloid beta protein antibody cross reactivities confound Alzheimer disease research? *J. Negat. Results Biomed.* 16, 1.
- Jarosz-Griffiths, H.H., et al., 2016. Amyloid- $\beta$  receptors: the good, the bad, and the prion protein. *J. Biol. Chem.* 291, 3174–3183.
- Jin, M., et al., 2011. Soluble amyloid  $\beta$ -protein dimers isolated from Alzheimer cortex directly induce Tau hyperphosphorylation and neuritic degeneration. *Proc. Natl. Acad. Sci. U. S. A.* 108 (14), 5819–5824.
- Kakio, A., et al., 2002. Interactions of amyloid beta-protein with various gangliosides in raft-like membranes: importance of GM1 ganglioside-bound form as an endogenous seed for Alzheimer amyloid. *Biochemistry* 41, 7385–7390.
- Kemble, G., et al., 1993. GPI- and transmembrane-anchored influenza hemagglutinin differ in structure and receptor binding activity. *J. Cell Biol.* 122, 1253–1265.
- Klingauf, J., et al., 1998. Kinetics and regulation of fast endocytosis at hippocampal synapses. *Nature* 394, 581–585.
- Klyubin, I., et al., 2008. Amyloid- $\beta$  protein dimer-containing human CSF disrupts synaptic plasticity: prevention by systemic passive immunization. *J. Neurosci.* 28, 4231–4237.
- Lauren, J., et al., 2009. Cellular prion protein mediates impairment of synaptic plasticity by amyloid- $\beta$  oligomers. *Nature* 457, 1128–1132.
- Lingwood, D., et al., 2008. Plasma membranes are poised for activation of raft phase coalescence at physiological temperature. *Proc. Natl. Acad. Sci. U. S. A.* 105, 10005–10010.
- Lipton, A.M., et al., 2001. Contribution of asymmetric synapse loss to lateralizing clinical deficits in frontotemporal dementias. *Arch. Neurol.* 58, 1233–1239.
- Masliah, E., et al., 1991. Cortical and subcortical patterns of synaptophysin-like immunoreactivity in Alzheimer's disease. *Am. J. Pathol.* 138, 235–246.
- Maxfield, F.R., Tabas, I., 2005. Role of cholesterol and lipid organization in disease. *Nature* 438, 612–621.
- McDonald, J.M., et al., 2010. The presence of sodium dodecyl sulphate-stable A $\beta$  dimers is strongly associated with Alzheimer-type dementia. *Brain* 133, 1328–1341.
- McLean, C.A., et al., 1999. Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. *Ann. Neurol.* 46, 860–866.
- Mehta, P.D., et al., 2000. Plasma and cerebrospinal fluid levels of amyloid beta proteins 1–40 and 1–42 in Alzheimer disease. *Arch. Neurol.* 57, 100–105.
- Montine, T.J., et al., 1999. Elevated CSF prostaglandin E2 levels in patients with probable AD. *Neurology* 53, 1495–1498.
- Nicoll, A.J., et al., 2013. Amyloid- $\beta$  nanotubes are associated with prion protein-dependent synaptotoxicity. *Nat. Commun.* 4.
- Parodi, J., et al., 2010.  $\beta$ -Amyloid causes depletion of synaptic vesicles leading to neurotransmission failure. *J. Biol. Chem.* 285, 2506–2514.
- Reddy, P.H., et al., 2005. Differential loss of synaptic proteins in Alzheimer's disease: implications for synaptic dysfunction. *J. Alzheimers Dis.* 7, 103–117.
- Robinet, P., et al., 2010. A simple and sensitive enzymatic method for cholesterol quantification in macrophages and foam cells. *J. Lipid Res.* 51, 3364–3369.
- Rudd, P.M., et al., 2001. Prion Glycoprotein: Structure, Dynamics, and Roles for the Sugars. 40. pp. 3759–3766.
- Sanchez-Mejia, R.O., et al., 2008. Phospholipase A<sub>2</sub> reduction ameliorates cognitive deficits in a mouse model of Alzheimer's disease. *Nat. Neurosci.* 11, 1311–1318.
- Selkoe, D.J., 2002. Alzheimer's disease is a synaptic failure. *Science* 298, 789–791.
- Shankar, G.M., et al., 2007. Natural oligomers of the Alzheimer amyloid- $\beta$  protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. *J. Neurosci.* 27, 2866–2875.
- Shankar, G.M., et al., 2008. Amyloid- $\beta$  protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nat. Med.* 14, 837–842.
- Shelat, P.B., et al., 2008. Amyloid beta peptide and NMDA induce ROS from NADPH oxidase and AA release from cytosolic phospholipase A<sub>2</sub> in cortical neurons. *J. Neurochem.* 106, 45–55.
- Solfrosi, L., et al., 2004. Cross-linking cellular prion protein triggers neuronal apoptosis in vivo. *Science* 303, 1514–1516.
- Stahl, N., et al., 1987. Scrapie prion protein contains a phosphatidylinositol glycolipid. *Cell* 51, 229–240.
- Stahl, N., et al., 1992. Glycosylinositol phospholipid anchors of the scrapie and cellular prion proteins contain sialic acid. *Biochemistry* 31, 5043–5053.
- Stuermer, C.A.O., et al., 2004. PrP<sup>C</sup> capping in T cells promotes its association with the lipid raft proteins reggie-1 and reggie-2 and leads to signal transduction. *FASEB J.* 14, 1731–1733.
- Suzuki, K.G.N., et al., 2007. GPI-anchored receptor clusters transiently recruit Lyn and G $\alpha$  for temporary cluster immobilization and Lyn activation: single-molecule tracking study 1. *J. Cell Biol.* 177, 717–730.
- Suzuki, K.G.N., et al., 2012. Transient GPI-anchored protein homodimers are units for raft

- organization and function. *Nat. Chem. Biol.* 8, 774–783.
- Tanzi, R.E., 2005. The synaptic A $\beta$  hypothesis of Alzheimer disease. *Nat. Neurosci.* 8, 977–979.
- Um, J.W., et al., 2012. Alzheimer amyloid-beta oligomer bound to postsynaptic prion protein activates Fyn to impair neurons. *Nat. Neurosci.* 15, 1227–1235.
- Walsh, D.M., et al., 2002. Naturally secreted oligomers of amyloid  $\beta$  protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* 416, 535–539.
- West, E., et al., 2017. The cholesterol ester cycle regulates signalling complexes and synapse damage caused by amyloid- $\beta$ . *J. Cell Sci.* 130, 3050–3059.
- Willem, M., et al., 2015.  $\eta$ -Secretase processing of APP inhibits neuronal activity in the hippocampus. *Nature* 526, 443–447.
- Yang, T., et al., 2017. Large soluble oligomers of amyloid  $\beta$ -protein from Alzheimer brain are far less neuroactive than the smaller oligomers to which they dissociate. *J. Neurosci.* 37, 152–163.