Monomeric amyloid-β reduced amyloid-β oligomer-induced synapse damage in neuronal cultures

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Abstract

Alzheimer's disease is a progressive neurodegenerative disease characterised by the accumulation of amyloid-β (Aβ) in the brain. Aβ 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 Aβ-induced synapse damage was investigated in cultured neurons by measuring the loss of synaptic proteins. Soluble Aβ oligomers, derived from Alzheimer's-affected brains, caused the loss of cysteine string protein and synaptophysin from neurons. When applied to synaptosomes Aβ oligomers increased cholesterol concentrations and caused aberrant activation of cytoplasmic phospholipase A_2 ($cPLA_2$). In contrast, Aβ monomer preparations did not affect cholesterol concentrations or activate synaptic $cPLA_2$, nor did they damage synapses. The Aβ oligomer-induced aggregation of cellular prion proteins (PrP^C) at synapses triggered the activation of $cPLA_2$ that leads to synapse degeneration. Critically, Aβ monomer preparations did not cause the aggregation of PrP^C; rather they reduced the Aβ oligomer-induced aggregation of PrP^C. The presence of Aβ monomer preparations also inhibited the Aβ oligomer-induced increase in cholesterol concentrations and activation of $cPLA_2$ in synaptosomes and protected neurons against the Aβ oligomer-induced synapse damage. These results support the hypothesis that Aβ monomers are neuroprotective. We hypothesise that synapse damage may result from a pathological Aβ monomer:oligomer ratio rather than the total concentrations of Aβ within the brain.

Key words: amyloid-β; cholesterol, monomers, oligomers, phospholipase A_2, synapses

Abbreviations – Alzheimer's disease (AD), Amyloid Precursor Protein (APP), amyloid-β (Aβ), cellular prion protein (PrP^C), cytoplasmic phospholipase A_2 ($cPLA_2$), cysteine-string protein (CSP), enzyme-linked immunoassay (ELISA), fragment antigen-binding (Fab), glycosylphosphatidylinositol (GPI), monoclonal antibody (mAb), phospholipase A_2-activating peptide (PLAP), prostaglandin (PG), sodium dodecyl sulphate (SDS), standard deviation (SD), vesicle-associated membrane protein (VAMP).

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-β (Aβ) 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 (Aβ) within the brain is thought to cause the synapse dysfunction and the memory loss that is characteristic of AD. Aβ 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 Aβ 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 Aβ. 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 Aβ derived from the brains Alzheimer's patients caused synapse degeneration in neurons (Yang et al., 2017). The biological effects of these Aβ 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 Aβ monomers that are found in high concentrations in the brain are poorly understood; while some studies report that Aβ 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 Aβ in monomer preparations, in addition to $A\beta_{40}$ and $A\beta_{42}$ 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 Aβ monomer preparations and Aβ oligomers were isolated from soluble brain extracts and their biological activity studied.

Increasing evidence suggests that aberrant activation of cytoplasmic phospholipase A_2 ($cPLA_2$) plays an important role in Aβ oligomer-induced synapse damage. For example, Aβ activates $cPLA_2$ (Anfuso et al., 2004; Shelat et al., 2008) and pharmacological inhibition of $cPLA_2$ protects cultured neurons against Aβ-induced synapse damage (Bate et al., 2010). In addition, $cPLA_2$ inhibitors ameliorated cognitive decline in a mouse model of AD (Sanchez-Mejia et al., 2008). Here we demonstrate that whereas Aβ oligomers increased synaptic cholesterol concentrations, activated $cPLA_2$ and caused synapse damage in cultured neurons, Aβ monomers had none of those effects. The cellular prion protein

(PrP^C) mediates Aβ-induced memory defects (Lauren et al., 2009) and aggregation of PrP^C by Aβ oligomers caused the activation of $cPLA_2$ and synapse damage (Bate and Williams, 2011). Here we report that Aβ monomers did not cause aggregation of PrP^C; rather that they reduced the aggregation of PrP^C by Aβ oligomers. The presence of Aβ monomers significantly reduced the Aβ oligomer-induced activation of $cPLA_2$ and synapse damage.

Materials and Methods

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 x 10^5 cells/well in 48 well plates that had been coated with poly-L-lysine. After 2 hours, 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 μM thiazolyl blue tetrazolium bromide for 3 hours at 37°C. The supernatant was removed, the formazan product solubilized in 200 μl 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 Aβ preparations for 24 hours. For other studies, neurons were pre-treated with Aβ monomers or control medium for 1 hour and then incubated with Aβ oligomers or a phospholipase A_2-activating protein (PLAP) (Bachem) for 24 hours. Treated neurons were washed 3 times with PBS and homogenised 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 x *g* for 5 minutes).

Western Blotting - Samples were mixed with Laemmli buffer containing β-mercaptoethanol, heated to 95°C for 5 minutes and proteins were separated by electrophoresis on 15% polyacrylamide gels. Proteins were transferred onto a Hybond-P polyvinylidiene fluoride membrane by semi-dry blotting. Membranes were blocked using 10% milk powder; synapsin-1 was detected with goat polyclonal (Santa Crux 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.

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 hour 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.

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 hour 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.

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 x *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 × *g* for 30 minutes at 4°C. The synaptosomes were collected from the interface between the 15% and 23% Percoll and washed (16,000 × *g* for 10 minutes at 4°C) and suspended in neurobasal medium containing B27 components at a concentration equivalent to 5 x 10^6 neurons per ml. All synaptosomes were used on the same day of preparation. Synaptosomes were incubated with Aβ monomers or Aβ oligomers for 1 hour at 37°C. In one experiment synaptosomes were pre-treated with Aβ monomers for 30 minutes and then incubated with Aβ oligomers for 1 hour 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)).

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 μM FM1-43 and 1 μM ionomycin, a calcium ionophore used to stimulate neurotransmitter release, for 5 minutes, washed 5 times in ice cold PBS and homogenised 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.

Cholesterol content - The concentrations of cholesterol in samples were measured using the Amplex Red cholesterol assay kit (Life Technologies) (Robinet et al., 2010). Briefly, control and treated synaptosomes were washed (400 × *g*, 10 min) and homogenized in 500 µL isopropanol:NP40 (9:1, v/v) and sonicated in a waterbath (30 minutes). Samples were centrifuged (10,000 × *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 (± cholesterol esterase)) were added and incubated at 37°C for 30 minutes. 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.

Activated cPLA$_2$ ELISA/Prostaglandin (PG)E$_2$ ELISA - The activation of cPLA$_2$ 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 µg/ml of the mouse mAb anti-cPLA$_2$, clone CH-7 (Upstate) and blocked with 5% milk powder. Samples were incubated for 1 hour and the amount of bound activated cPLA$_2$ was detected using a rabbit polyclonal anti-phospho-cPLA$_2$ (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$_2$ present were expressed as "units activated cPLA$_2$" where 1 unit was defined as the amount of activated cPLA$_2$ in control samples. The amounts of PGE$_2$ in synaptosomes were determined using a competitive enzyme immunoassay kit (R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

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 minutes (Disruptor genie, Scientific

Instruments). This process was performed 3 times before tubes were centrifuged at 16,000 x *g* for 10 minutes to remove particulate matter. Soluble material was prepared by passage through a 50 kDa filter (Sartorius) (16,000 x *g* for 30 minutes to remove proteolytic enzymes, membrane-bound and larger forms of Aβ. 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 Aβ" preparations were used in this study was that natural Aβ oligomers and monomers (unlike synthetic peptides) are stable (Jin et al., 2011; Shankar et al., 2008; Walsh et al., 2002). For example, after Aβ monomers had been incubated for 7 days at 37ºC in a cell free system we did not find any Aβ oligomers. Brain extracts were not pooled, rather monomers and oligomers were isolated from 3 main brain extracts and each were measured in triplicate.

Monomer and Oligomer preparations – Monomer preparations were prepared by passage through a 10 kDa filter (Sartorius). In theory Aβ dimers have a molecular weight of 9 kDa and should pass through the 10 kDa filter. However, natural Aβ is bound to membrane components that increase the molecular weight of Aβ dimers and consequently no Aβ dimers were detected in 10 kDa filtrates. It should be noted that Aβ monomer preparations contained $A\beta_{40}$ and $A\beta_{42}$ (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/minute. Preparations were stored at -80ºC. 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 polyvinylidiene fluoride membrane by semi-dry blotting and blocked using 10% milk powder. Aβ was detected by incubation with mAb 6E10, reactive with amino acids 1-16 of human Aβ (Covance), biotinylated anti-mouse IgG, extravidin-peroxidase and enhanced chemiluminescence.

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

Sample preparation for $A\beta_{40}/A\beta_{42}$ ELISA – To detach $A\beta_{42}$ from membrane components that blocked specific epitopes samples (300 µl) were mixed with 700 µl of propan-2-ol and sonicated. Proteins were precipitated by adding 250 µls 100%w/v trichloroacetic acid, incubating on ice for 30 mins and centrifugation (16,000 x *g* for 10 mins at 4°C). The pellet was washed twice with ice cold acetone, suspended, first in 1% NH_4OH and then in a buffer containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.2% SDS and sonicated.

$A\beta_{40}/A\beta_{42}$ ELISA - Nunc Maxisorb immunoplates were coated with mAb 4G8 (epitope 17-24) (Covance) and blocked with 5% milk powder. Samples were added for 1 hour. In separate plates, $A\beta_{40}$ was detected with rabbit polyclonal PC-149 (Merck) and $A\beta_{42}$ 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 $A\beta_{1-40}/A\beta_{1-42}$ (Bachem) suspended in the sample preparation buffer (as above) and sonicated.

PrP^C filtration assays – Soluble PrP^C 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^C was incubated with Aβ monomers or Aβ oligomers for 1 hour and then centrifuged through 50 kDa filters (Vivaspin - Sartorius) (Bate and Williams, 2011). The filtrate was collected and tested for the presence of PrP^C by ELISA.

PrP^C ELISA - The amount of PrP^C 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).

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.

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).

Results

Aβ 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 Aβ on the amounts of synaptic proteins in cultured neurons was studied. A soluble brain extract from an AD patient contained forms of Aβ including monomers, dimers and trimers (Figure 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 (Figure 1B). However, there was no loss of caveolin from treated neurons, nor was there any reduction in cell viability (98% survival ± 5 compared with 100% survival ± 6, n=0, P=0.45), indicating synapse degeneration without significant neuronal death. Immunodepletion with monoclonal antibody (mAb) 6E10 (reactive with Aβ, amino acids 1-16) reduced the concentrations of both $A\beta_{40}$ (7.44 nM ± 0.8 SD to 0.5 nM ± 0.4 nM) and $A\beta_{42}$ (1.21 nM ± 0.16 to 0.16 nM ± 0.09 nM) in brain extracts, whereas mock-depletion had no significant effect on $A\beta_{40}$ (7.44 nM ± 0.8 to 7.12 nM ± 0.65 nM) or $A\beta_{42}$ (1.21 nM ± 0.22 to 1.09 nM ± 0.29 nM). The Aβ-depleted brain extract still contained other peptides as shown by a coomassie blue stain (supplementary Figure 1). The addition of brain extracts to neurons resulted in the dose-dependent loss of synaptophysin (Figure 1C) and CSP (Figure 1D) (Bate and Williams, 2011). The addition of a brain extract that had been depleted of Aβ did not affect the amounts of synaptophysin or CSP in neurons; observations consistent with the hypothesis that Aβ 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).

Removal of Aβ monomers increased the brain extract-induced synapse damage – Brain extracts from AD patients contain a mixture of Aβ monomers and soluble Aβ oligomers, predominantly dimers and trimers (Klyubin et al., 2008; Mc Donald et al., 2010; Shankar et al., 2008). Aβ oligomers were prepared by passage of the extract through a 10 kDa filter; the retained material contained Aβ oligomers but lacked smaller APP fragments that we have called Aβ monomers (Figure 2C, lane 2). Brain extracts depleted of Aβ monomers caused more synapse damage, as measured by the loss of synaptophysin (Figure 2A) and CSP (Figure 2B), when compared with the original brain extracts that contained Aβ monomers (Figure 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 (Figure 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.

Aβ monomers did not cause synapse damage – Aβ monomers were prepared by passing brain extracts through a 10 kDa filter. This preparation contained only Aβ monomers (Figure 3A, lane 2) which eluted in a single peak from a Superdex 75 PC column (Figure 3B). The addition of Aβ monomer preparations containing 1 nM $A\beta_{42}$ did not affect the amounts of synaptophysin (Figure 3C) or CSP (Figure 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 units synaptophysin ± 3.5 compared with 100 units ± 3.8, n=12, P=0.34). In contrast, Aβ 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.

Aβ 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 minute increased the amounts of FM1-43 taken up by synaptosomes in a dose-dependent manner (Figure 4A). The addition of Aβ 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 (Figure 4B). Immunodepletion studies showed that Aβ 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 (Figure 4C). Aβ oligomers, but not Aβ monomer preparations caused a dose-dependent inhibition of the ionomycin-induced uptake of FM1-43 (Figure 4D).

Aβ monomers inhibited Aβ oligomer-induced synapse damage – The hypothesis that Aβ monomers protected synapses was tested. Pre-treatment with monomer preparations (containing 5 nM $A\beta_{42}$, approximately 5 times the concentration of Aβ oligomers that caused maximum synapse damage (1 nM)) protected neurons against the loss of synaptophysin (Figure 5A) and CSP (Figure 5B) triggered by Aβ 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) (Figure 5C). Immunodepletion studies were used to examine the nature of the neuroprotective moiety in the monomer preparations. Immunodepletion with mAb 6E10 (reactive with Aβ, amino acids 1-16) reduced the concentrations of $A\beta_{42}$ (from 5 nM ± 0.2 to 0.1 nM ± 0.1 nM, n=12, $P<0.01$) whereas mock-depletion had no significant effect (5 nM ± 0.2 compared with 4.9 nM ± 0.3 nM, n=12, P=0.27). Neurons pre-treated with the Aβ-depleted monomer preparations were not protected against synapse

damage caused by Aβ oligomers (containing 0.5 nM $A\beta_{42}$) (Figure 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 $A\beta_{42}$ (Figure 5E).

Aβ monomers inhibited Aβ 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 Aβ-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 $cPLA_2$ found in synaptosomes (Figure 6A) whereas brain extracts depleted of Aβ had a reduced effect. Aβ oligomers, but not Aβ monomers, activated synaptic $cPLA_2$ (Figure 6B). Pre-treatment of synaptosomes with monomer preparations (containing 1 nM $A\beta_{42}$, approximately 5 times the concentration of Aβ oligomers that caused maximum activation of $cPLA_2$ (0.2 nM)) reduced the activation of $cPLA_2$ by Aβ oligomers (Figure 6C) but not by PLAP (Figure 6D) indicating that monomers did not have a direct effect upon this enzyme. Immunodepletion studies demonstrated that it was Aβ within the monomer preparations that was responsible for blocking the Aβ oligomer-induced activation of synaptic $cPLA_2$ (Figure 6E) and the production of prostaglandin (PG)E_2 (Figure 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 Aβ monomers that inhibit the Aβ oligomer-induced activation of synaptic $cPLA_2$ and PGE_2 production.

Aβ monomers inhibited the Aβ oligomer-induced increase in cholesterol concentrations - The concentrations 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) (Figure 7A) an observation that is consistent with reports of increased cholesterol in Aβ positive synapses in the cortex of Alzheimer's patients (Gylys et al., 2007). This effect of brain extracts was lost after the removal of Aβ. Further studies showed that Aβ oligomers increased synaptic cholesterol while monomers did not (Figure 7B). When incubated with brain extracts containing between 1 and 0.06 nM $A\beta_{42}$ there were significant correlations between concentrations of cholesterol and activated $cPLA_2$ (Figure 7C) and PGE_2 (Figure 7D). Next, the effects of Aβ monomers on the Aβ oligomer-induced increase in synaptic cholesterol concentrations were studied. Monomer preparations (containing 5 nM $A\beta_{42}$) blocked the increase in cholesterol concentrations caused by Aβ oligomers (containing 0.5 nM $A\beta_{42}$) (Figure 7E). Immunodepletion studies showed that Aβ monomers were responsible for blocking the Aβ oligomer-induced increase in cholesterol.

Aβ monomers inhibited Aβ oligomer-induced aggregation of PrP^C – The observations that cross-linkage of PrP^C by antibodies leads to neurodegeneration (Solforosi et al., 2004) and that Aβ oligomers cause PrP^C aggregation (Bate and Williams, 2011) suggested that aggregation of PrP^C is a critical factor that initiates synapse damage and the pathogenesis of AD. Here we show that PrP^C incubated with brain extract formed complexes containing at least two PrP^C molecules as shown by immunoblot (Figure 8A). This qualitative study was supported by quantitative studies in which 1 nM soluble PrP^C was passed through a 50 kDa filter as described (Bate and Williams, 2011). Although PrP^C has a molecular weight that is dependent upon glycosylation it does not exceed 37 kDa and consequently all soluble PrP^C passed through a 50 kDa filter. PrP^C incubated with Aβ monomers (1 nM $Aβ_{42}$) also passed through a 50 kDa filter whereas incubation with Aβ oligomers (1 nM $Aβ_{42}$) reduced the amounts of PrP^C that passed through a 50 kDa filter by approximately 80%. The formation of PrP^C complexes of greater than 50 kDa was lost after the removal of Aβ from brain extracts (Figure 8B) indicating that Aβ oligomers cross-link PrP^C. To test the hypothesis that the binding of Aβ monomers to PrP^C block the aggregation of PrP^C by Aβ oligomers, soluble PrP^C was pre-treated with monomers (containing 1 nM $Aβ_{42}$) and incubated with oligomers (containing 1 nM $Aβ_{42}$). Pre-treatment of soluble PrP^C with Aβ monomers significantly reduced the Aβ oligomer-induced formation of PrP^C complexes greater than 50 kDa (Figure 8C).

Fab fragments block the mAb-mediated cross-linkage of PrP^C and synapse damage – The cross-linkage of PrP^C by mAbs caused neurodegeneration *in vivo* (Solforosi et al., 2004) and synapse damage in cultured neurons (Bate and Williams, 2011). The PrP^C-reactive mAb 4F2 mimicked some of the effects of Aβ oligomers on synaptosomes, including increasing cholesterol concentrations (Figure 9A) and activating $cPLA_2$ (Figure 9B). These effects were not seen in synaptosomes incubated with monovalent Fab fragments derived from 4F2 that do not cross-link PrP^C. When the mAb/Fab fragments were incubated with cultured neurons, the divalent mAb 4F2 caused a dose-dependent loss of synaptophysin (Figure 9C) and CSP (Figure 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 (Figure 10A) and the mAb 4F2-induced activation of $cPLA_2$ (Figure 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 (Figure 10C) and CSP (Figure 10D) from neurons.

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^C and the subsequent increase in synaptic cholesterol concentrations and the activation of $cPLA_2$.

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 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 $Aβ_{42}$ 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 $Aβ_{42}$) 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 $Aβ_{42}$ (Figure 3C). We note that in addition to $Aβ_{40}$ and $Aβ_{42}$ 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 $Aβ_{40}$, $Aβ_{42}$ or other APP fragments sharing this epitope.

Recent studies suggest that Aβ caused synapse damage as a consequence of aberrant cell signaling. As synthetic $Aβ_{42}$ 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 $cPLA_2$ in synaptosomes, consistent with reports that pharmacological inhibition of $cPLA_2$ protected cultured neurons against Aβ-induced synapse damage (Bate et al., 2010) and ameliorated cognitive decline in a mouse model of AD (Sanchez-Mejia et al., 2008). The activation of $cPLA_2$ by Aβ involves the translocation of $cPLA_2$ to lipid rafts and is sensitive to cholesterol depletion (Bate and Williams, 2011). Here we demonstrate that Aβ oligomers, but not Aβ monomers, increased cholesterol concentrations and activated $cPLA_2$ within synapses.

PrP^C that is highly expressed at synapses (Brown, 2001; Herms et al., 1999) has been identified as a receptor for $Aβ_{42}$ (Balducci et al., 2010; Lauren et al., 2009) and the activation of $cPLA_2$ in synapses by Aβ involves PrP^C (Bate and Williams, 2011). PrP^C 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^C 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 (Eggeling 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^C caused by Aβ 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 Aβ oligomers, but not monomers, cause PrP^C to aggregate, increase synaptic cholesterol concentrations and activate $cPLA_2$. This hypothesis is supported by our observations that the cross-linkage of PrP^C by mAb 4F2 mimicked some of the effects of Aβ oligomers upon synaptosomes, including increasing cholesterol concentrations and activating $cPLA_2$; mAb 4F2 also caused synapse degeneration in cultured neurons. In contrast, no changes in synaptic cholesterol concentrations, activation of $cPLA_2$ or synapse damage were observed in synaptosomes or neurons incubated with monovalent Fab fragments derived from mAb 4F2. The reorganisation 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^C can cause the clustering of receptor-associated signalling molecules including $cPLA_2$ within lipid raft platforms. It should be noted that Aβ has been reported to bind to numerous other proteins (Jarosz-Griffiths et al., 2016) meaning that Aβ oligomers have the potential to link PrP^C with other proteins. The heterogeneity of complexes formed between Aβ oligomers and numerous different "receptors" may help to explain how Aβ causes the wide variety of cellular responses reported.

We report that Aβ monomer preparations protected neurons against the Aβ oligomer-induced synapse damage. These monomers also reduced the Aβ oligomer-induced increase in synaptic cholesterol concentrations and activation of $cPLA_2$ that is closely associated with synapse damage. Aβ monomers did not affect the activation of $cPLA_2$ by PLAP, nor did they affect PLAP-induced synapse damage, indicating that they interfere with a stimulus-specific pathway. Aβ monomers did not cause aggregation of PrP^C, consistent with the hypothesis that Aβ contains a single PrP^C-binding site and consequently Aβ oligomers, but not Aβ monomers cause the aggregation of PrP^C. The presence of Aβ monomers blocks the Aβ oligomer-induced aggregation of PrP^C which has been shown to cause cell signalling and synapse degeneration (Bate and Williams, 2012). These results contrast studies that showed that synthetic $Aβ_{42}$ 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 Aβ 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 (Butikofer et al., 2001; Elfrink et al., 2008; Kemble et al., 1993). For example, native PrP^C contains sialic acid (Rudd et al., 2001; Stahl et al., 1992) a glycan implicated in Aβ binding and structure (Hong et al., 2014; Kakio et al., 2002).

Critically we found that pre-treatment with Aβ monomer preparations prevented the Aβ oligomer-induced aggregation of PrP^C. These preparations contain a mixture of Aβ species and consequently it was not possible to identify the precise form of Aβ that is responsible for this effect. It is possible that more than one Aβ species is involved. We hypothesise that Aβ monomers compete with Aβ oligomers for binding sites on PrP^C. Thus, the binding of Aβ monomers to PrP^C prevented the binding of Aβ oligomers and subsequent cross-linkage of PrP^C. This hypothesis is supported by studies using Fab fragments of the PrP^C-binding mAb 4F2. Pre-treatment of synaptosomes with monovalent Fab fragments of 4F2 (that do not cross-link PrP^C) significantly reduced both mAb 4F2-induced increase in cholesterol concentrations and mAb 4F2-induced activation of $cPLA_2$. 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 Aβ fragment, oligomer size and the conformation of Aβ. Although many studies measure Aβ levels as a marker of disease progression, they often do not differentiate between Aβ monomers and oligomers. These results suggest that the concentration of Aβ monomers is another factor that should be considered in determining disease progression. Thus, the presence of high concentrations of Aβ monomers could protect neurons against synapse damage even in the presence of high concentrations of Aβ oligomers. However, the role of Aβ monomers is not completely clear as others have reported that the presence of Aβ monomers was associated with dementia (Mc Donald et al., 2010). It should be noted that this work was carried out using human brain-derived Aβ 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.

Conclusions

In conclusion these results support the hypothesis that Aβ monomers have a protective role by reducing synapse damage caused by Aβ oligomers. They are consistent with the hypothesis that Aβ monomers compete with Aβ oligomers for binding sites on PrP^C and that the binding of Aβ monomers prevents the Aβ oligomer-induced aggregation of PrP^C that triggers aberrant activation of $cPLA_2$ and synapse damage. These observations suggest that AD may develop as a result of changes in the Aβ monomer:oligomer ratio.

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.

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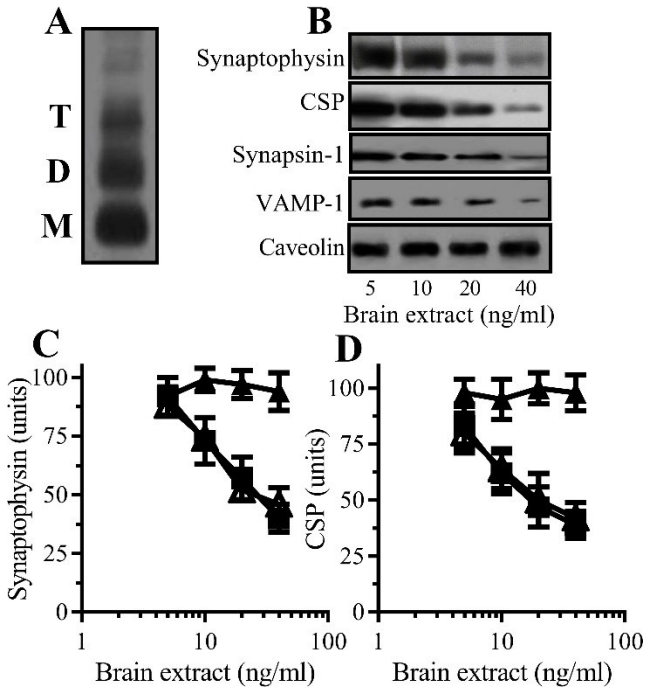

Figure 1 – Soluble brain extracts caused Aβ-dependent synapse damage – (A) Western blot showing forms of Aβ including monomers (M), dimers (D) and trimers (T) contained within a soluble brain extract. (B) Western blots showing the amounts of synaptophysin, CSP, synapsin-1, VAMP-1 and caveolin in cultured neurons that had been incubated with brain extract as shown. The amounts of synaptophysin (C) and CSP (D) neurons incubated with brain extract (■), Aβ-depleted brain extract (▲) or mock-depleted brain extract (△) as shown for 24 hours. Values are means ± 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 (■).

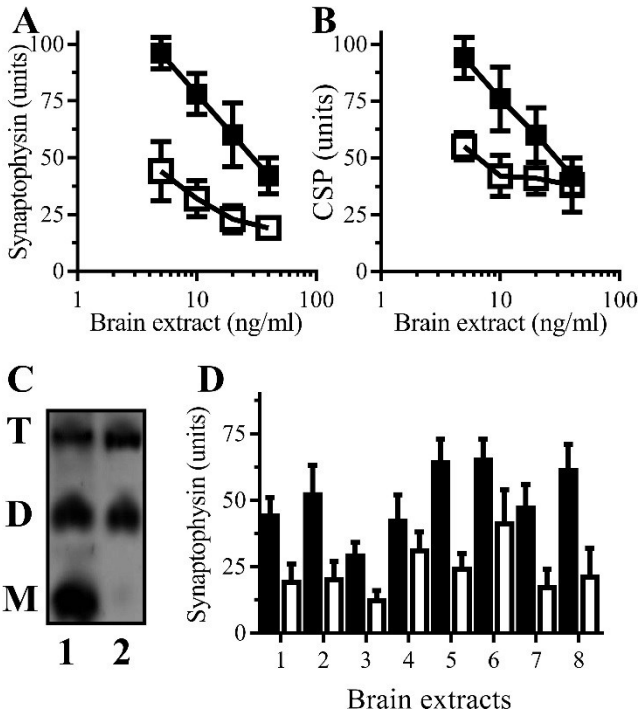

Figure 2 – Removal of Aβ monomers from brain extracts increases synapse damage – The amounts of synaptophysin (A) and CSP (B) in neurons incubated with soluble brain extract (■) or monomer-depleted brain extract (□) as shown. Values are means ± SD from triplicate experiments performed 4 times, n=12. (C) Immunoblot showing Aβ monomers (M), dimers (D) and trimers (T) contained within brain extract (1) and brain extract retained by a 10 kDa filter (Aβ 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 ± SD from triplicate experiments performed twice, n=6.

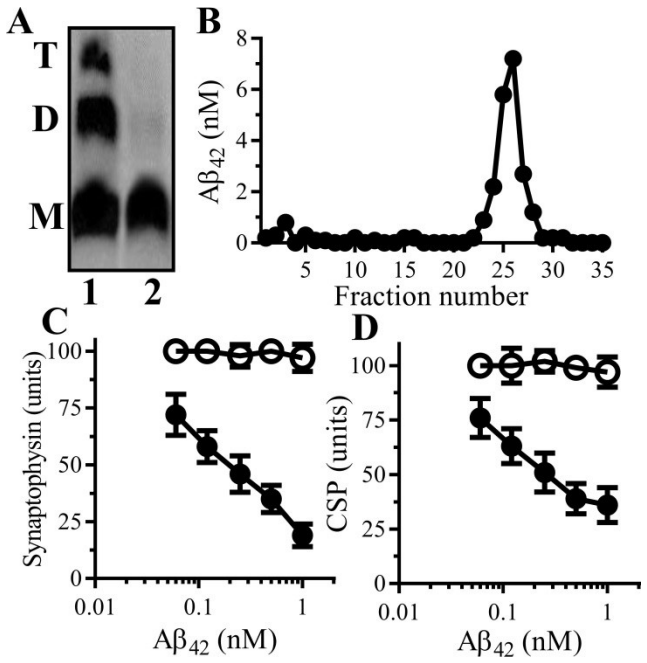

Figure 3 – Aβ monomers do not cause synapse damage - (A) Immunoblot showing forms of Aβ including monomers (M), dimers (D) and trimers (T) in soluble brain extract (1) and brain extract passed through a 10 kDa filter (Aβ monomers) (2). (B) The concentrations of Aβ in fractions eluted from a Superdex 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 ± SD from triplicate experiments performed 3 times, n=9.

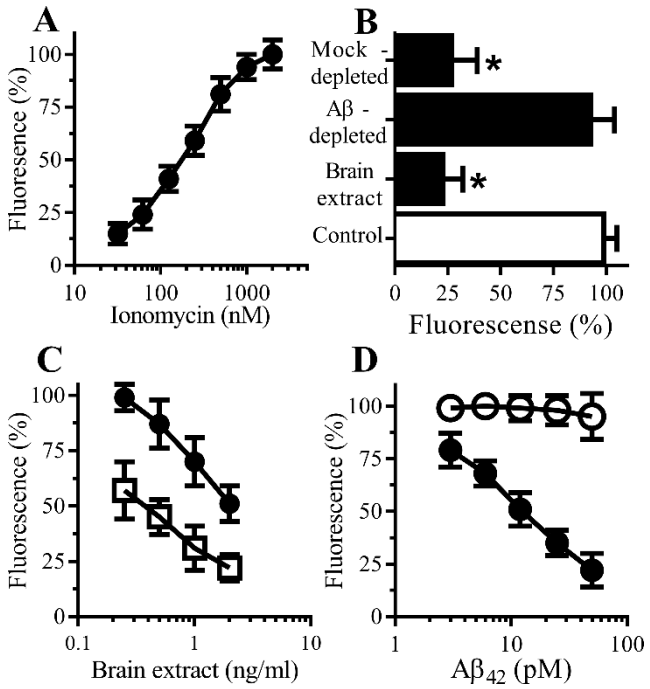

Figure 4 – Aβ oligomers inhibit synaptic vesicle recycling – (A) The amounts of FM1-43 in synaptosomes incubated with ionomycin as shown (●). Values are mean % fluorescence (100% = maximum fluorescence) ± SD from triplicate experiments performed 3 times (n=9). (B) The amounts of FM1-43 in synaptosomes incubated with control medium (□), brain extract, Aβ-depleted brain extract or mock-depleted brain extract (■) for 30 minutes and incubated with 1 μM ionomycin. Values are means ± 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 minutes and incubated with 1 μM ionomycin. Values are means ± SD from triplicate experiments performed 3 times (n=9). (D) The amounts of FM1-43 in synaptosomes incubated with oligomer (●) or monomer (○) preparations containing $A\beta_{42}$ for 30 minutes as shown and incubated with 1 μM ionomycin. Values are means ± SD from triplicate experiments performed 3 times (n=9).

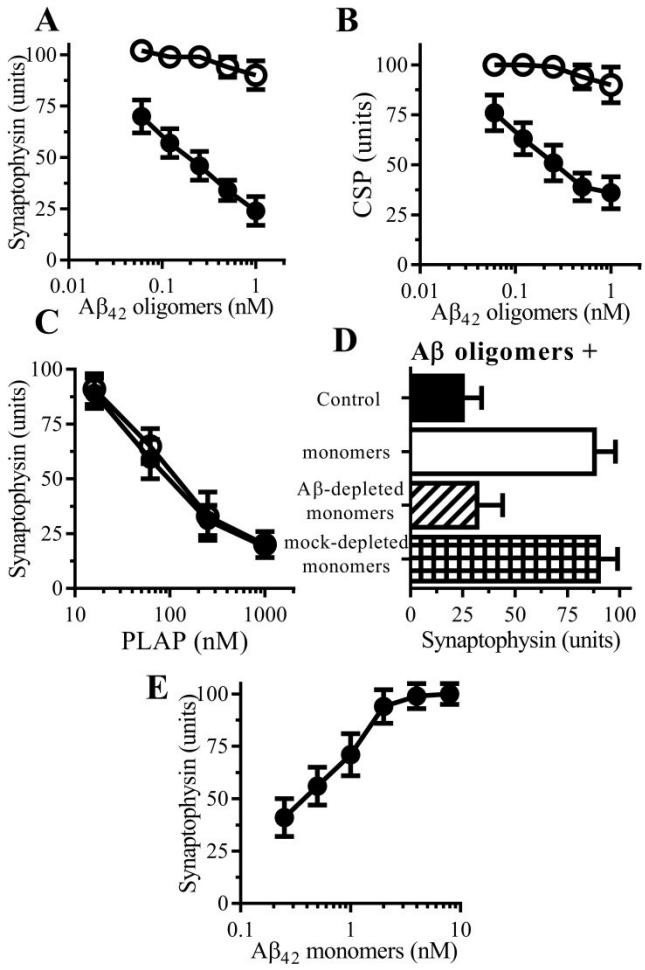

Figure 5 - Aβ monomers inhibit Aβ oligomer-induced synapse damage - The amounts of synaptophysin (A) and CSP (B) in neurons pre-treated with control medium (●) or monomers (containing 5 nM $A\beta_{42}$) (○) and incubated with Aβ oligomers as shown. Values are means ± SD from triplicate experiments performed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

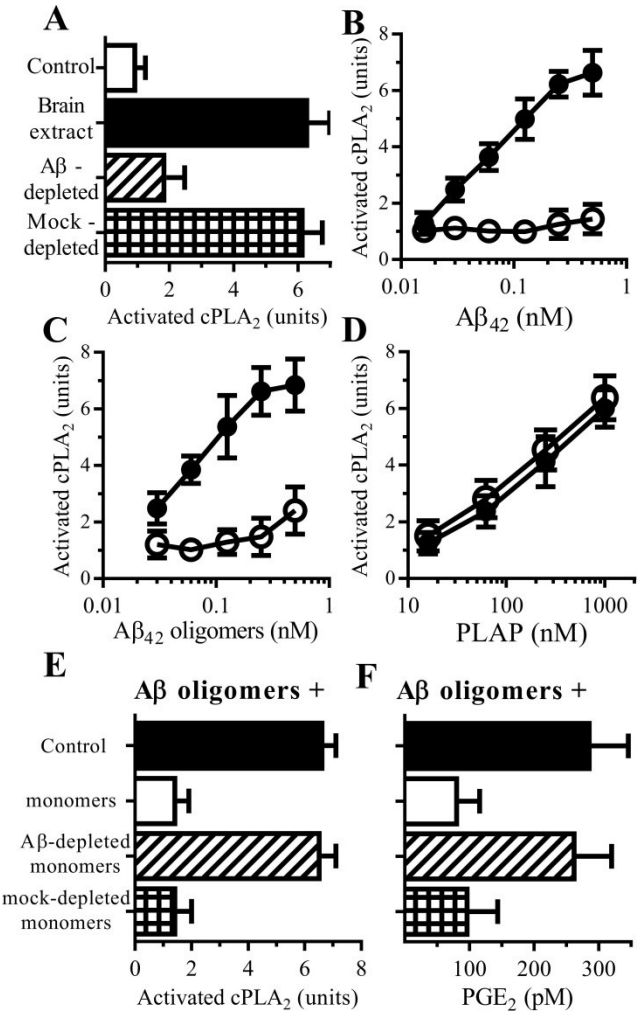

Figure 6 - Aβ monomers inhibit the Aβ oligomer-induced activation of synaptic cPLA$_2$ - (A) The amounts of activated cPLA$_2$ in synaptosomes incubated with control medium (□) brain extract (■), Aβ-depleted brain extract (striped bar) or mock-depleted brain extract (hatched bar). Values are means ± SD from triplicate experiments performed 3 times, n=9. (B) The amounts of activated cPLA$_2$ in synaptosomes incubated with oligomers (●) or monomers (○) containing Aβ$_{42}$ as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9. The amounts of activated cPLA$_2$ in synaptosomes pre-treated with control medium (●) or monomers containing 1 nM Aβ$_{42}$ (○) and incubated with oligomers containing Aβ$_{42}$ (C) or PLAP (D) as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9. The amounts of activated cPLA$_2$ (E) and PGE$_2$ (F) in synaptosomes pre-treated with control medium (■), monomers containing 1 nM Aβ$_{42}$ (□), Aβ-depleted monomers (striped bars) or mock-depleted monomers (hatched bars) and incubated with oligomers containing 0.5 nM Aβ$_{42}$.Values are means ± SD from triplicate experiments performed 3 times, n=9.

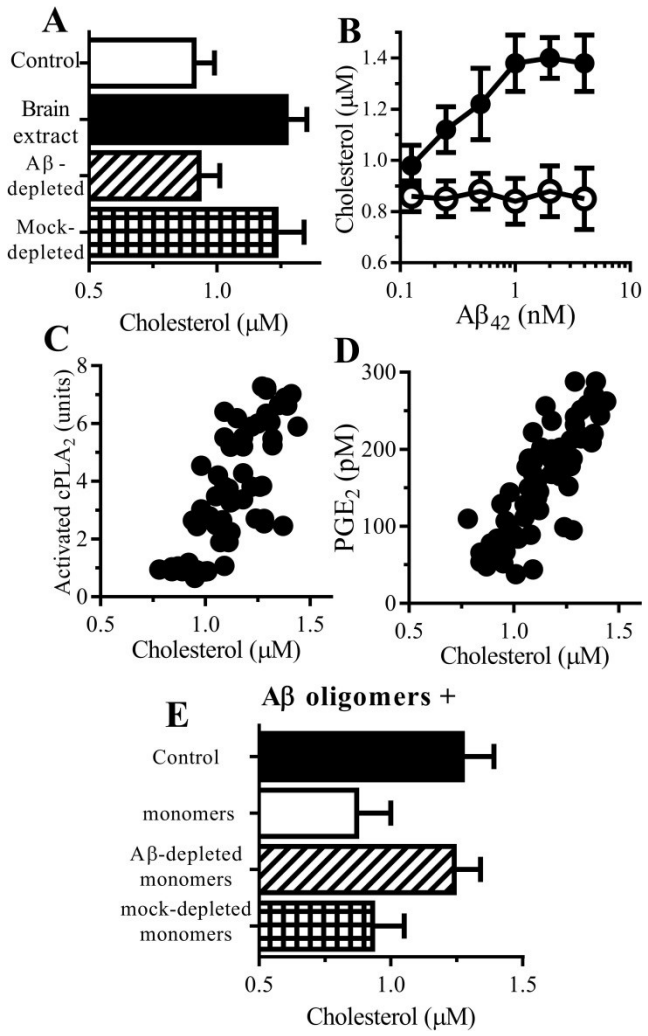

Figure 7. Aβ monomers inhibit the Aβ oligomer-induced increase in synaptic cholesterol - (A) The concentrations of cholesterol in synaptosomes incubated with control medium (□) brain extract (■), Aβ-depleted brain extract (striped bar) or mock-depleted brain extract (hatched bar). Values are means ± SD from triplicate experiments performed 3 times, n=9. (B) The concentrations of cholesterol in synaptosomes incubated with oligomers (●) or monomers (○) containing $A\beta_{42}$ as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9. In synaptosomes incubated with oligomers containing between 0.06 and 1 nM $A\beta_{42}$ there were significant correlations between the concentrations of cholesterol and the amounts of activated $cPLA_2$ (C), Pearson's coefficient=0.75, p <0.01 and the concentrations of PGE_2 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 $A\beta_{42}$. Values are means ± SD from triplicate experiments performed 3 times (n=9).

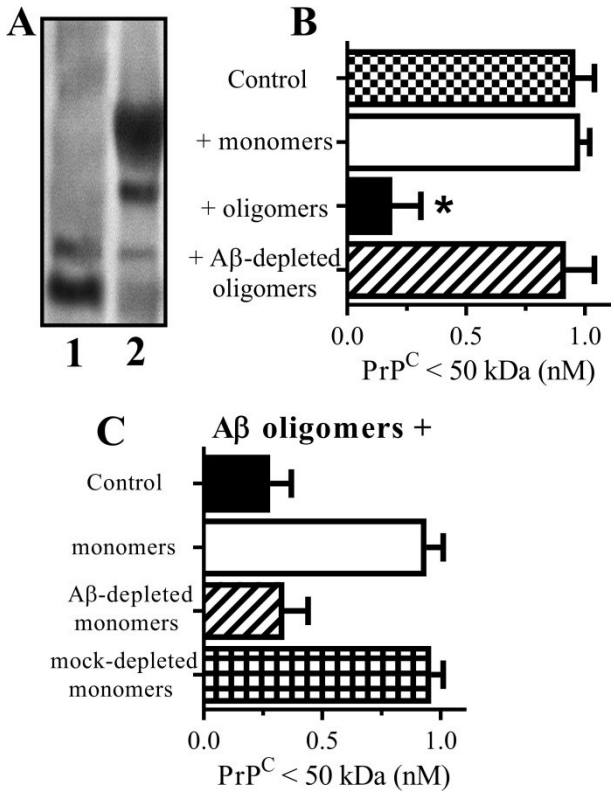

Figure 8 - Aβ oligomers cause aggregation of PrP^C – (A) Immunoblot showing PrP^C in preparations of (1) (1) PrP^C or (2) PrP^C mixed with Aβ oligomers separated by non-denaturing gel electrophoresis. (B) The concentrations of PrP^C passing through a 50 kDa filter after incubation with control medium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

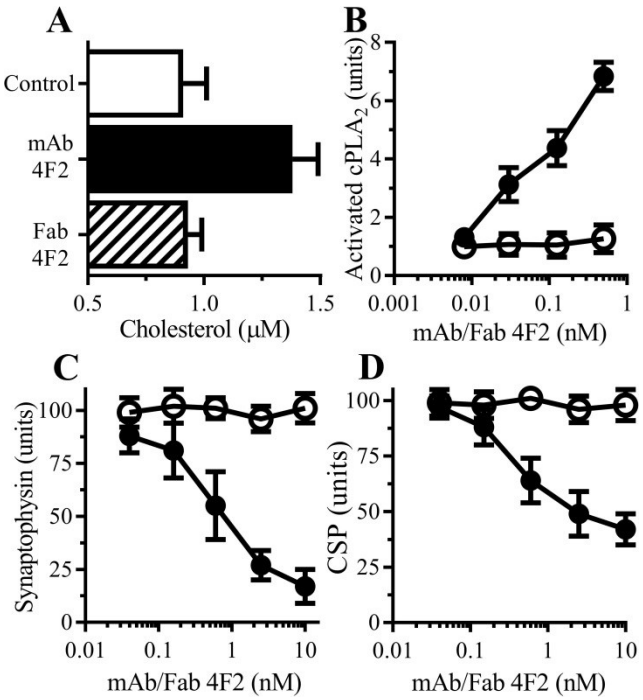

Figure 9 – A PrP^C mAb increases synaptic cholesterol, activates synaptic $cPLA_2$ and triggers synapse damage – (A) The concentrations of cholesterol in synaptosomes treated with control medium (□), 1 nM mAb 4F2 (■) or 2 nM 4F2 Fab fragments (striped bar). Values are means ± SD from triplicate experiments performed 3 times (n=9). *=cholesterol significantly higher than in control synaptosomes. (B) The amounts of activated $cPLA_2$ 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.

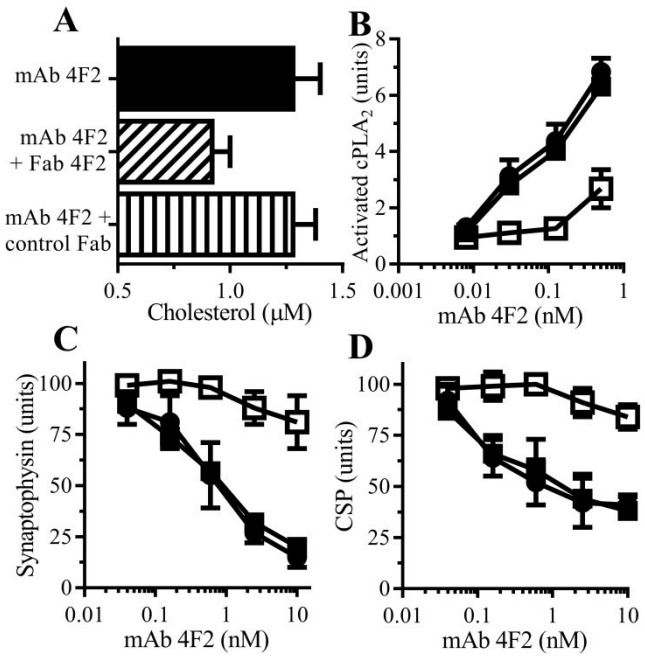

Figure 10 – Fab 4F2 fragments protect neurons against mAb 4F2-induced synapse damage – (A) The concentrations of cholesterol in synaptosomes incubated with 2 nM mAb 4F2 (■), or mixtures containing 2 nM 4F2 Fab fragments and 2 nM mAb 4F2 (striped bar) or 2 nM control Fab fragments and 2 nM mAb 4F2 (vertical striped bar). Values are means ± SD from triplicate experiments performed 3 times (n=9). *=cholesterol significantly lower than in synaptosomes incubated with mAb 4F2. (B) The amounts of activated $cPLA_2$ 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 ± 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 ± SD from triplicate experiments performed 3 times, n=9.