Molecular BioSystems

PAPER

View Article Online
View Journal | View Issue

Cite this: *Mol. BioSyst.*, 2017, **13**, 1545

Phosphorylation of a full length amyloid-β peptide modulates its amyloid aggregation, cell binding and neurotoxic properties

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Received 27th April 2017,
Accepted 29th May 2017

DOI: 10.1039/c7mb00249a

rsc.li/molecular-biosystems

Amyloid beta peptide (Aβ) is the major protein component of the amyloid plaques that are present in the brains of Alzheimer's disease (AD) patients. Aβ42 peptide is a known neurotoxic agent that binds to neurons and, under specific aggregation conditions, triggers cell death. Aβ peptide can undergo specific amino acid posttranslational modifications, such as phosphorylation, that are important for modulating its proteolytic degradation, aggregation, binding to lipid membranes and neurotoxic functions. Peptides phosphorylated at serine 8 in full-length Aβ42 (pAβ42) were synthesised and compared to native Aβ42 peptide. Their secondary structures, aggregation properties and interactions with plasma membranes of primary cortical neurons were investigated. The results revealed that pAβ42 has increased β-sheet formation with rapid amyloid formation in a synthetic lipid environment, which was associated with increased cellular binding but concomitant diminished neurotoxicity. Our data support the notion that phosphorylation of Aβ42 promotes the formation of amyloid plaques in the brain, which lack the neurotoxic properties associated with oligomeric species causing pathogenesis in AD.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that has a devastating impact on our aging population as it causes clinical memory loss, confusion, cognitive deficits and brain atrophy.[1] A major pathological hallmark of AD is the extracellular accumulation of amyloid-β (Aβ) peptide as insoluble amyloid plaques, an event that precedes the formation of the intraneuronal neurofibrillary tangles (composed of hyperphosphorylated tau) in the brain which lead to significant synaptic damage and neuronal cell death.[1,2] Thus, while phosphorylation of the tau protein dramatically alters its properties, little is known about how phosphorylation of Aβ modulates its biological properties. A full-length Aβ peptide has 42 amino acids and there are three potential amino acid phosphorylation sites – serine at positions 8 and 26 and tyrosine at position 10.[3] Phosphorylated Aβ (pAβ) has been detected in brain samples from APP transgenic mice[4,5] and in human brain,[5] brain plaques and blood samples from patients diagnosed with AD,[6] while human cerebrospinal fluid has been shown to exhibit Aβ phosphorylating activity.[7] Further analysis revealed that pAβ accumulation in the parenchyma and vasculature deposits was mainly found in late-stage AD brains.[8] In addition, a positive correlation between the soluble pAβ concentration and angiotensin converting enzyme activity in the parahippocampal cortex, but not in the midfrontal cortex, was found, suggesting that a positive feedback mechanism exists between pAβ and the enzyme.[8] Phosphorylation also decreased Aβ proteolytic degradation and clearance by microglial cells.[9] Taken together, phosphorylation of Aβ therefore has a number of important roles in promoting AD pathogenesis, including promoting aggregation and decreasing its proteolytic clearance.

The ability of Aβ to bind lipids and form amyloid plaques indicates that its interaction with neuronal membranes is a key event for its aggregation into toxic species to induce neurotoxicity.[10–12] Thus, it is important to understand whether posttranslational modifications of Aβ can modulate its interaction with lipid membranes, lead to the peptide acquiring cytotoxic properties and cause cell death. Since phospholipids and cholesterol are the main components of membranes,[13] the interactions of Aβ peptide with membrane lipids and lipid mimetic compounds were studied. However, in order to further understand the effect of phosphorylation, we chemically synthesised full-length native Aβ42 and pAβ42 with the serine phosphorylated (pSer) at position 8 and examined their interactions with lipid

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membranes using both a synthetic model membrane and mouse primary cortical cultures.

Results

Synthesis of full-length phosphorylated Aβ42 peptides

We determined the optimised conditions for synthesising and purifying Aβ42 and pAβ42 peptides. For the preparation of pAβ42 peptide, while the whole chain was synthesised using 5 minutes of microwave coupling at 86 °C, some residues were coupled at room temperature for extended periods of time (up to 1 h). This was to minimise de-phosphorylation, racemization or aspartimide formation, particularly for the coupling of pSer, aspartic acid, histidine or cysteine amino acids. The amino acids were assembled on commercially available alanine loaded solid phase Wang resin. The advantage of this resin is that it swells efficiently in the solvent used for coupling and phosphorylation. We determined the optimal conditions for the elution solvent (B: Isp : ACN : Milli-Q = 40 : 40 : 20; A: 0.1 M ammonium acetate buffer pH 9.2; gradient of B: 20–50% in 30 min) and used a high temperature (50 °C) to purify the Aβ42 and pAβ42 peptides because of their very hydrophobic and aggregating properties. For the synthesis of the AlexaFlour labelled peptides, the serine residue at position 26 was replaced with a cysteine residue using a previously reported in-house procedure.[14] Chromatograms and mass spectra analyses are shown in Fig. 1.

Amyloid aggregation formation of pAβ42 in a lipid environment

Aggregation of Aβ42 and pAβ42 was detected using a ThT fluorescence assay, which is routinely used for the identification and quantification of amyloidogenic β-sheet structural formation *in vitro*.[15] The ThT fluorescence profile of the pAβ42 peptide was similar to that of the native Aβ42 peptide when suspended in PBS buffer only, displaying the typical initial lag phase and then undergoing rapid aggregation and reaching maximal fluorescence at around 12 h (Fig. 2A). In the presence of POPS/POPC large unilamellar vesicles (LUVs), the ThT fluorescence intensity for the pAβ42 peptide showed a slower aggregation profile and plateauing at 12 h again, while the ThT fluorescence intensity was at least double that of the native Aβ42 peptide (Fig. 2B). The inclusion of cholesterol in the POPS/POPC LUVs (POPS/POPC/Chol) resulted in initial rapid aggregation of the pAβ42 at t = 0 and slow and consistent aggregation over the 24 h incubation period, and the ThT fluorescence intensity was again at least double that of the native Aβ42 peptide over the whole incubation period (Fig. 2C).

Secondary structure of pAβ42 in a lipid environment

CD spectroscopy analysis was done to determine whether the secondary structure and folding properties of the phosphorylated Aβ42 peptide were modified in a PBS buffer environment and in the presence of synthetic lipid membrane model systems (Fig. 3). The results showed that the Aβ42 peptide in PBS buffer was mainly unstructured (Fig. 3A) while pAβ42 adopted more β-sheet configuration (higher intensity ~200 nm and less intensity near ~215 nm) and random coil configuration (Fig. 3B). The addition of POPS/POPC LUVs to these peptides resulted in a changed confirmation for both peptides. For Aβ42, there was a small increase in the β-sheet structure while the addition of POPS/POPC/Chol LUVs led to a much higher intensity of random coil structure (Fig. 3A). In contrast, the addition of the POPS/POPC LUVs had only a small effect on pAβ42 conformation showing some extended β-sheet structure but the presence of cholesterol in POPS/POPC/Chol LUVs had little effect on the secondary structure, which may be due to the faster aggregation or stronger interaction of this modified peptide with model membranes.

Toxicity and cell binding properties of phosphorylated Aβ42

Having confirmed that the secondary structure and amyloid formation properties of pAβ42 differed from the native Aβ42,

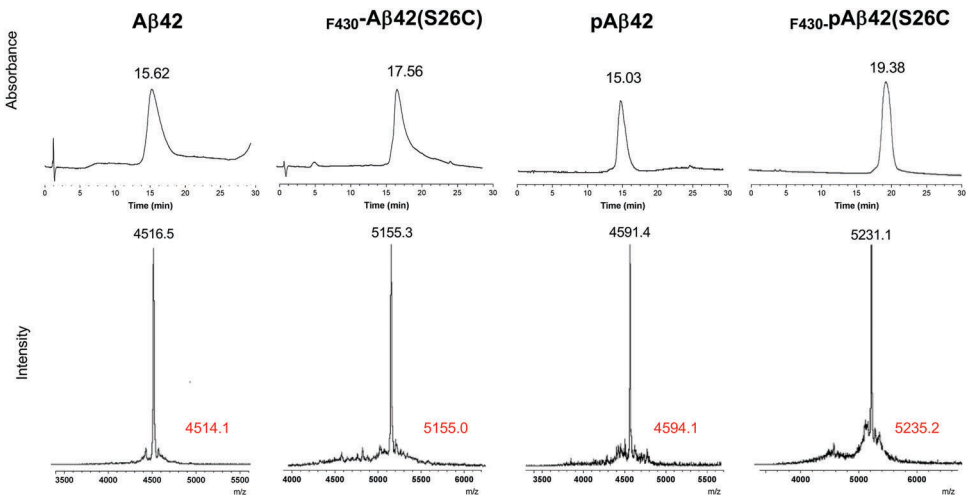

Fig. 1 Characterisation of purified synthesised Aβ42 peptides by chromatographic analysis. RP-HPLC (top panel) displaying peak elution times and MALDI TOF MS trace analysis (bottom panel) displaying calculated (black) and observed (red) mass values for each peptide synthesised.

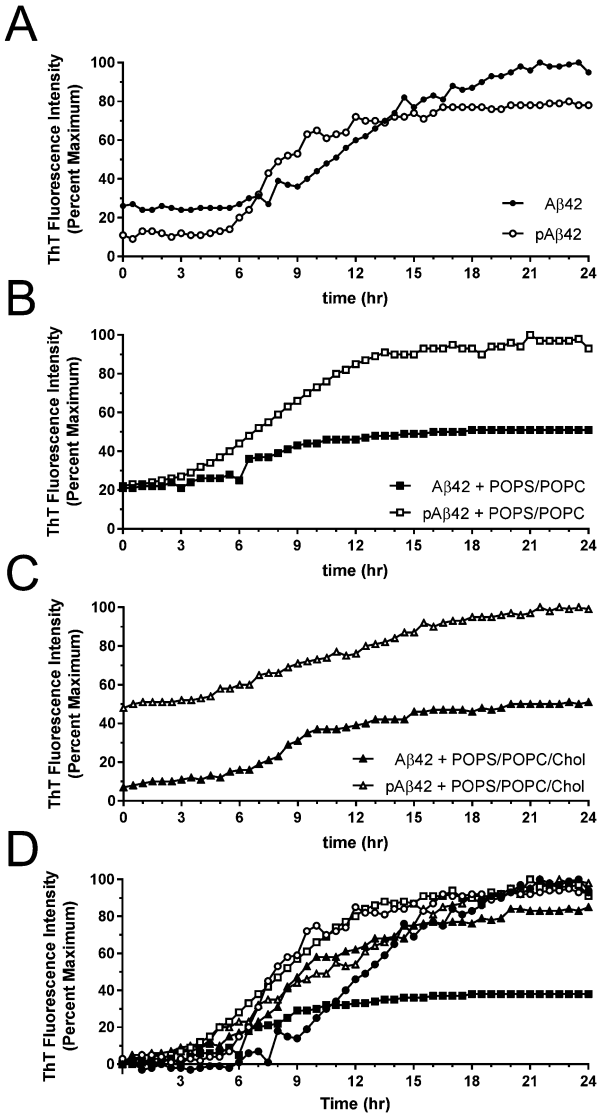

Fig. 2 Detection of amyloid structures by Thioflavin T (ThT) assay. Synthetically prepared Aβ42 (filled symbols) and pAβ42 (open symbols) peptides (10 μM) were analysed in the presence of (A) PBS buffer only (circles), (B) PBS buffer containing POPC/POPS LUVs (squares), and (C) PBS buffer containing POPC/POPS/Chol (triangles), at a peptide : lipid ratio of 1 : 30. The data were normalised to the maximum ThT level in the respective panels and (D) t = 0 subtracted. Traces are representative of 3 different experiments.

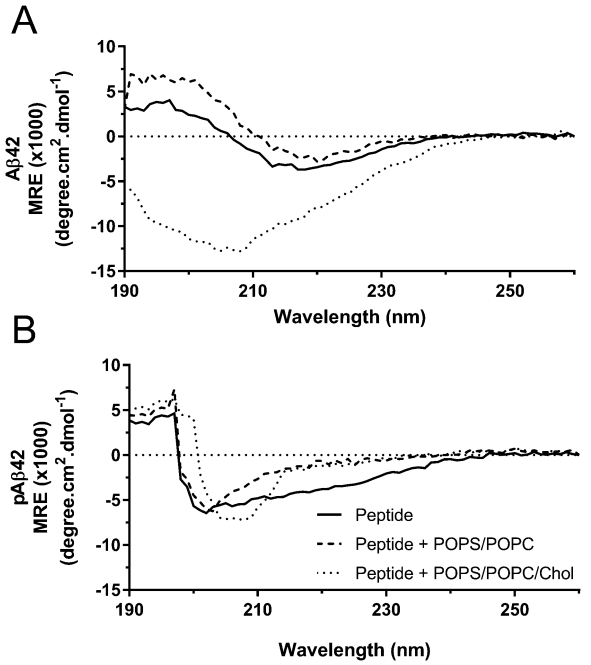

Fig. 3 The secondary structure of the synthesised Aβ peptides was determined by CD spectral analysis. The CD spectral traces for (A) Aβ42 and (B) pAβ42 peptides (10 μM) were compared in solutions containing PBS buffer only (solid black line), PBS buffer containing POPC/POPS LUVs (long dashed black line), and PBS buffer containing POPC/POPS/Chol LUVs (dotted black line) at a peptide : lipid ratio of 1 : 30. Traces are representative of 3 different experiments.

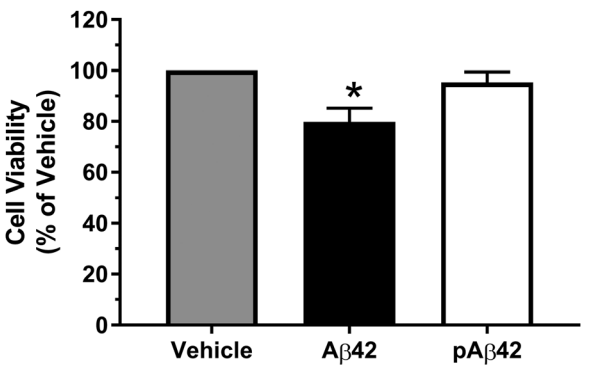

Fig. 4 Neurotoxicity of the synthesised Aβ42 and pAβ42 peptides. Primary cortical neurons were grown at a density of 150 000 cells per cm^2 for 6 days *in vitro* and the viability of these neuronal cultures was determined following 15 μM peptide treatment for 96 h by CCK assay. The data were normalised and expressed as a percentage of the vehicle treated cultures. The results are mean ± SEM. Statistical comparison between vehicle and treatment groups was done using the Students t-test. *, $p > 0.05$ *vs.* vehicle treated cells. Experiments were done in triplicate and repeated 3 times.

we then tested the toxicity of these peptides in primary mouse cortical cultures. As expected, treating the cortical cultures for 4 days with native Aβ42 induced a significant reduction in cell viability at 15 μM (Fig. 4). By contrast, pAβ42 displayed no neurotoxicity at the same concentration (Fig. 4).

Since Aβ neurotoxicity is associated with neuronal cell binding,[11,16,17] we examined whether the non-toxic nature of pAβ42 may be due to an altered cell binding characteristic. This experiment was performed using F_{430}-Aβ42(S26C) and F_{430}-pAβ42(S26C) labelled peptides at a subtoxic concentration (5 μM). The labelled peptides were added to the primary cortical cultures for 24 h and cell binding was examined histologically using identical microscope and image acquisition settings (Fig. 5). As expected, the native F_{430}-Aβ42(S36C) peptide bound to the extracellular structures of the neuron in a punctate matter, as described previously[11,16,17](Fig. 5A), while the F_{430}-pAβ42(S36C) peptide similarly bound to the plasma membrane surface of neuronal cell structures (Fig. 5B). The amount of cell surface binding was quantitated by identifying neuronal cell structures using an anti-Tau antibody, and the cell images were imported

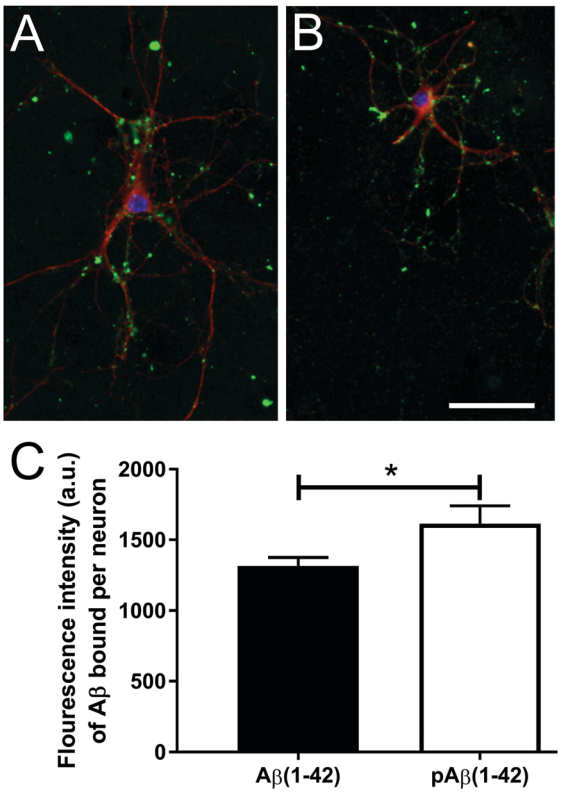

Fig. 5 Quantitation of Aβ binding to cortical neurons. Neurons at 6DIV (150 000 cells per cm^2) were treated for 24 h with 5 μM of: (A) F_{430}-Aβ42(S26C) and (B) F_{430}-pAβ42(S26C) (green); and (C) The amount of F_{430}-Aβ peptide binding to neurons was determined by histological analysis, as described in the Experimental section, and the calculated cell fluorescence intensity is graphed. Neurons were co-stained for Tau (red) and nucleus using DAPI (blue). $N = 3$. The results are mean ± SEM. *, $p < 0.05$ *vs.* F_{430}-Aβ42(S26C) treated cells.

into an image analysis software program. The regions of interest around the neuronal cellular structures were identified and the fluorescence intensity levels of the Aβ bound peptide molecules (*i.e.*, green channel) were measured, background regions subtracted and adjustments made to account for the number of neurons per image. The results show that the F_{430}-pAβ42(S36C) peptide had a significantly higher fluorescence intensity compared to the native F_{430}-Aβ42(S36C) peptide (Fig. 5C).

Discussion

This study reveals that posttranslational phosphorylation at Ser8 of full-length Aβ42 causes significant changes to its biophysical, neurotoxic and neuron cell binding properties. This is the first report using in-house peptide chemistry to synthesise a *bona fide* full-length peptide with site specific phosphorylation at Ser8 (pAβ42 peptide). One of the major challenges of investigating the Aβ peptide is its synthesis and purification, since the full-length Aβ42 peptide is very hydrophobic and, therefore, known as a very "difficult peptide" to make.[18] The major challenges associated with the synthesis of this peptide are due to its high content of hydrophobic residues that are present in the C-terminal sequence, which makes solid phase synthesis of this peptide very challenging even without additional posttranslational modifications to specific amino acids.[19,20] Previous reports have investigated shorter Aβ peptide sequences with phosphorylation at Ser8, pAβ(1-16)[21] and pAβ(1-40),[5,9,22] and Ser26 pAβ(1-40),[23] which were purchased from commercial suppliers. Alternatively, phosphorylated Aβ peptides were prepared by incubating synthetic Aβ peptides with enzymes, such as protein kinase A,[5] which readily phosphorylated S8 in Aβ40, and human cdc2 kinase,[24] which bound to and induced phosphorylation purportedly at Ser26 for the Aβ25-35, Aβ40 and Aβ42 peptides. We now provide the optimised conditions for successfully chemically synthesising and purifying full-length pAβ42 peptides, as well as preparing F_{430}-Aβ42(S26C) peptides with greater than 96% purity.

The post-translational modifications of Aβ peptide that are responsible for alterations to its secondary structure and aggregation propensity, particularly upon interaction with a lipid membrane, are contributing factors to the development of this AD pathogenesis. Our findings reveal that the pAβ42 peptide aggregated in a similar manner to its native Aβ42 peptide in an aqueous environment, but in the presence of phospholipid POPS/POPC LUVs or POPS/POPC/Chol LUVs, the pAβ42 peptide aggregated more rapidly and to a much higher extent compared to the native peptide. These results are in agreement with the pAβ40(S8) peptide, which also displayed a similar higher rate and amount of ThT reactive aggregate material compared to the native Aβ40 peptide[5,22] while aggregation of pAβ40(S26)[23] remained largely unchanged with only a slight increase in ThT absorbance after 30 h of incubation in a lipid-free model experiment. This increased aggregation of pAβ42 was confirmed by the CD spectroscopy data, which revealed increased β-sheet formation in the presence of POPS/POPC/Chol LUVs (Fig. 3). Phosphorylation at Ser8 for Aβ40 led to the characteristic pattern of extended β-sheet structure only when it was allowed to aggregate for at least 2 h while in its monomeric state.[5] We and others show that pAβ is largely in a disordered state.[5,22,23] The combined *in vitro* data suggest that a lipid environment is an important parameter for the promotion and formation of amyloid structures for the Aβ peptide.

We have previously shown that Aβ peptide binding to neuronal cultures is a key determinant for inducing cell toxicity, with the notable key exception of the D-handed peptide, which while binding to neurons in culture was not toxic.[11,16,17,25,26] We observed a higher level of F_{430}-pAβ42(S26C) binding compared to the F_{430}-Aβ42(S26C) peptide, which may be explained by the CD (Fig. 3) and ThT (Fig. 2) results, which showed that the pAβ42 peptide had a higher propensity to aggregate and bind to model plasma membranes. The higher cell binding for F_{430}-pAβ42(S26C) did not translate into increased cell toxicity (Fig. 4); rather, we saw diminished toxicity of the pAβ42 peptide in our primary cortical cell culture model. Our results are in agreement with the results found for the p(S26)Aβ40 peptide, which also was not toxic to mouse primary cortical cultures.[23] However, the same laboratory found that the p(S8)Aβ40 peptide was toxic to human iPSC-derived neurons and p(S26)Aβ40 was toxic to both human neuroblastoma cells and iPSC-derived

neuronal cultures.[27] In addition, an *in vivo Drosophila* model, where a pseudo phosphorylated Aβ was prepared with an S8D mutation, was found to strongly promote age-dependent degeneration of the photoreceptor cells in the eye of the Drosophila compared to native Aβ.[5] Taken together, our results support the notion that phosphorylation of Aβ promotes its rapid aggregation into non-toxic amyloid structures that are readily found on the extracellular membrane structures in the brain and represent the pathological hallmark for AD diagnosis.

Conclusion

Synthetic preparation of a full-length Aβ42 peptide incorporating a phosphorylated Ser8 amino acid was found to promote aggregation into non-toxic amyloid structures, especially when in contact with a lipid membrane. Our results reveal that pAβ42 has increased β-sheet formation with rapid amyloid formation in a model phospholipid membrane environment, which is associated with increased cellular binding but concomitant diminished neurotoxicity. The observations that the aggregation of Aβ was substantially increased in the presence of pAβ seeds (*i.e.*: containing preformed oligomeric nuclei)[5] and that pAβ is mainly found in late-stage AD brains[8] in combination with our own cell binding data support the notion that the posttranslational phosphorylation of Aβ42 is an important event that promotes amyloid plaque formation in the brain, which lacks the neurotoxic properties associated with oligomeric species causing pathogenesis in AD.

Experimental

Materials

All chemicals and solvents were obtained from Sigma-Aldrich (Sydney, Australia) unless otherwise stated.

Peptide synthesis

The Aβ42 and pAβ42 peptides were synthesized using previously reported in-house procedures for peptide synthesis,[14] but a number of modifications and optimisation steps were required to achieve high levels of purity and yields. The peptides were synthesised using the Fmoc/*t*Bu solid phase strategy with microwave irradiation at 86 °C on a CEM Liberty synthesizer. The histidine, cysteine in the Aβ42 peptides and phosphoserine in the pAβ42 peptides were coupled without microwaving. Fmoc protected amino acids were used for peptide synthesis (GL Biochem, China). Peptides were cleaved using trifluoracetic acid (TFA), anisol, triisopropylsilane, and Milli-Q water (94 : 3 : 2 : 1) for the Aβ42 peptide; and TFA, anisol, triisopropylsilane, Milli-Q water, and thioanisole (90 : 2.5 : 2.5 : 2.5 : 2.5) for the pAβ42 peptide. The crude peptides, except pAβ42, were purified by RP-HPLC using a Phenomenex C4 column (particle size 5 μm, 4.6 × 150 mm), with a gradient of 10–90% acetonitrile (0.1% TFA) in 30 min. The pAβ42 peptides were purified using isopropanol : acetonitrile : Milli-Q water at a ratio of 40 : 40 : 20 and with 0.1 M ammonium acetate buffer of pH 9.2 with a gradient of 20–50% in 30 min. The purity (96 and 98%) and yields (18 and 14%, calculated from the crude starting material) were determined for Aβ42 and pAβ42, respectively.

To prepare the fluorescently labelled Aβ peptides, the serine residue (S) at position 26 in the peptide sequence was replaced with a cysteine residue (C). These Aβ42(S26C) and pAβ42(S26C) peptides were synthesized and labelled with AlexaFluor 430 as NHS ester forms (Invitrogen, Sydney, Australia) using thiol-maleimide conjugation chemistry.[14] The coupling reaction between NHS-AlexaFluor 430 (1.4 μmol, 1 mg) and *N*-(2-aminoethyl) maleimide (1.68 μmol, 0.43 mg) was performed in the presence of *N*,*N*-diisopropylethylamine (2.68 μmol, 0.05 μl) in dimethylformamide. Mal-AlexaFluor was used to react with the side chain of the cysteine residue. The final Aβ-AlexaFluor labelled peptides (termed F_{430}-Aβ42(S26C) and F_{430}-pAβ42(S26C), respectively; Fig. 1) were purified using the same conditions as described for the unlabelled peptide. For F_{430}-Aβ42 and F_{430}-pAβ42, respectively, the purities were 97 and 99% and the yields were 43 and 32% (calculated from purified Aβ42(S26C) peptides as the starting material).

Peptide preparation

Lyophilised peptides were prepared by dissolving the peptides in a sequence of buffers at a ratio of 2 : 7 : 1 v/v. 20 mM NaOH was added to the peptide and vortexed and sonicated in an ice chilled water bath for 15 min to fully solubilise the peptide. Milli-Q water and 10× PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 138 mM NaCl, 8 mM Na_2HPO_4, at pH 7.4) were added, the solubilised peptide was spun in a benchtop centrifuge at 16 000 *g* for 5 min (to remove amorphous aggregates) and the supernatant was transferred to a clean tube and stored on ice until used (typically within 1 h of its preparation). The final Aβ42 peptide concentrations were determined by UV absorption spectroscopy using the molar extinction coefficient of 75887 M^{-1} cm^{-1} at 214 nm.

Preparation of model lipid membranes

Multilamellar vesicles were prepared using previously reported in-house procedures with slight modifications.[10,17] Briefly, phospholipids were sourced from Avanti Polar Lipids (Alabaster, USA) and desired amounts of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) were combined together (POPC/POPS) at a 1 : 1 (w/w) ratio or with cholesterol (POPC/POPS/Chol) at a 1 : 1 : 1 (w/w) ratio and dissolved in a solution of chloroform and methanol at a 3 : 1 (v/v) ratio and the organic solvents were evaporated under vacuum to obtain a lipid film. The lipid film was placed under vacuum overnight to evaporate residual organic solvents. The dried film was resuspended in Milli-Q water, homogenised and extruded through a polycarbonate Whatman Nuclepore membrane (100 nm pore size) in 10 mM phosphate buffer pH 7.4 to produce large unilamellar vesicles (LUVs).

Thioflavin T assay

Thioflavin T (ThT) fluorescence was used to monitor the presence of β-sheet amyloidogenic structures of the Aβ peptides. ThT binds to aggregated Aβ peptides, which causes an increase in ThT fluorescence, as measured by excitation at 440 nm and

emission at 482 nm. Briefly, Aβ42 and pAβ42 peptides were prepared at 10 μM in the presence of 20 μM ThT in a 96 well microplate and incubated at 37 °C with orbital shaking at 700 rpm using a FLUOstar Omega plate reader (BMG Labteck, Melbourne, Australia) with 30 min readings taken over a 24 h period. The ThT assay was performed with 10 μM Aβ peptides in PBS buffer only (phosphate buffer, pH 7.4 and 1 mM NaCl), as well as in the presence of POPS/POPC LUVs and POPS/POPC/Chol LUVs at a peptide : lipid molar ratio of 1 : 30. All experimental combinations were performed at the same time.

CD spectroscopy

The peptide secondary structure and conformational changes upon the addition of LUV lipid systems were studied using circular dichroism (CD) spectroscopy. CD traces were acquired at room temperature using a Chirascan-plus instrument, a 1 cm quartz cuvette and instrument settings of 1 nm step size, 1 nm bandwidth and a wavelength range of 190 to 260 nm. CD measurements were performed with 10 μM Aβ peptides in PBS buffer only, as well as in the presence of the prepared LUVs and LUVs plus cholesterol at a peptide : lipid molar ratio of 1 : 30. All the experimental combinations were performed on the same day.

Primary neuronal cultures

Male and female 14-day-old embryonic mice were taken from pregnant female mice and used to prepare cortical neuronal cultures under sterile conditions, as described previously.[13] The mice were provided by the University of Melbourne, School of Biomedicine, Animal House Facility. These procedures were approved by a local institutional Animal Ethics Committee. Mouse cortical neuronal cultures were prepared under sterile conditions, as described previously,[16] and approved by a local institutional Ethics committee. Briefly, embryonic day 14 C57/BL6 mice cortices were removed, dissected free of meninges, and dissociated in 0.025% (w/v) trypsin in Krebs buffer. The dissociated cells were triturated using a filter-plugged fine pipette tip, pelleted, resuspended in plating media (minimum Eagle's media containing 10% fetal calf serum and 5% horse serum) and counted. Cortical neuronal cells were seeded at 150 000 cells per cm^2 for 2 h, then the plating media was replaced with freshly prepared neurobasal media containing B27 supplements, gentamicin, and 0.5 mM glutamine (NB/27). All the tissue culture reagents were purchased from Invitrogen, Melbourne, Australia, unless otherwise stated. Cells were seeded onto poly-D-lysine treated 48 well plates for cell viability assays, and onto 12 mm glass coverslips (placed in 24 well plates) for histological analysis. All cultures were maintained in a humidified incubator set at 37 °C supplemented with 5% CO_2. This method resulted in cultures highly enriched for neurons (>95% purity) with minimal astrocyte and microglial contamination.

Cell viability assays

The neuronal cells were allowed to mature for 6 days in culture before Aβ treatment was commenced. Freshly prepared soluble Aβ stock solutions were diluted to a final concentration of 15 μM in freshly prepared neurobasal medium containing B27 supplements (minus antioxidants) and then added to the neuronal cells for 4 days of treatment in the 37 °C incubator. Cell viability was quantitated using the CCK8-assay kit (Dojindo, Auspep, Melbourne, Australia) as previously described.[25] The data were normalized and calculated as a percentage of vehicle-treated cells.

Fluorescence histochemistry

The neuronal cells were allowed to mature for 6 days in culture before Aβ treatment was commenced. Freshly prepared soluble F_{430}-Aβ42(S26C) and F_{430}-pAβ42(S26C) stock solutions were diluted to a final concentration of 5 μM in freshly prepared neurobasal medium containing B27 supplements (minus antioxidants) then added to the neuronal cells for 24 h treatment in a 37 °C incubator. Cells were then washed with PBS buffer followed by washing with cold 0.1 M sodium carbonate pH 10 and fixed in 4% paraformaldehyde/PBS for 20 min before being incubated in permeabilization buffer (10% goat serum in PBS containing 0.01% Triton-X) for 20 min and then in blocking buffer (10% goat serum in PBS) for 60 min. Primary anti-Tau antibody (Rabbit polyclonal, Dako, Sydney, Australia) was diluted at 1 : 1000 in block buffer and incubated on the cells overnight at 4 °C. The cells were washed several times in PBS, and finally incubated in an anti-rabbit-Alexa567 secondary antibody (1 : 500 in block buffer) and DAPI (for the detection of the cell nucleus) for 60 min, washed in PBS then mounted onto glass slides using mounting media (Prolong Gold, Invitrogen). A Zeiss axioscope2 microscope using a 40X objective lens equipped with a Zeiss HRc camera with filter sets for FITC (green) and Rhodamine (red) was used to take images for histological binding analysis. Identical settings and exposure time were used to capture images for both peptides. The images were processed using Zen blue software (Zeiss, Germany) to generate tiff images before importing them into ImageJ software (NIH, version 1.5b),[28] and the area and integrated density parameter measurements were determined from the selected regions of interest from the images containing neuronal cell structures. The corrected total cell fluorescence intensity = [integrated density − (area of selected cell) × (mean fluorescence of background readings)].

Acknowledgements

The authors thank Dr J. Karas for his advice on peptide synthesis and purification. This work was funded by a grant from the National Health and Medical Research Council of Australia to GDC and seed grant funds from the Melbourne Neuroscience Institute, awarded to GDC, MAH and FS.

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