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Sialylated glycosylphosphatidylinositols suppress the production of toxic amyloid-β oligomers

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Abstract

The production of amyloid-β (Aβ) is a key factor driving pathogenesis in Alzheimer's disease (AD). Increasing concentrations of soluble Aβ oligomers within the brain lead to synapse degeneration and the progressive dementia characteristic of AD. Since Aβ exists in both disease-relevant (toxic) and non-toxic forms, the factors that affected the release of toxic Aβ were studied in a cell model. 7PA2 cells expressing the human amyloid precursor protein released Aβ oligomers that caused synapse damage when incubated with cultured neurons. These Aβ oligomers had similar potency to soluble Aβ oligomers derived from the brains of Alzheimer's patients. Although the conditioned media from 7PA2 cells treated with the cellular prion protein (PrP^C) contained Aβ it did not cause synapse damage. The loss of toxicity was associated with a reduction in Aβ oligomers and an increase in Aβ monomers. The suppression of toxic Aβ release was dependent upon the glycosylphosphatidylinositol (GPI) anchor attached to PrP^C and treatment of cells with specific GPIs alone reduced the production of toxic Aβ. The efficacy of GPIs was structure dependent and the presence of sialic acid was critical. The CM from GPI-treated cells protected neurons against Aβ oligomer-induced synapse damage; neuroprotection was mediated by Aβ monomers. These studies support the hypothesis that the ratio of Aβ monomers to Aβ oligomers is a critical factor that regulates synapse damage.

Keywords - amyloid, glycosylphosphatidylinositol, neuroprotection, prion, sialic acid, synapse

Short Title – Glycosylphosphatidylinositols reduce toxic amyloid-β production

Abbreviations - Alzheimer's disease (AD), amyloid-β (Aβ), amyloid precursor protein (APP), Chinese hamster ovary (CHO), conditioned media (CM), cysteine string protein (CSP), detergent-resistant membranes (DRMs), glycosylphosphatidylinositol (GPI), high performance thin layer chromatography (HPTLC), monoclonal antibody (mAb), platelet activating factor (PAF), prion protein (PrP)**,** *Ricinus Communis* Agglutinin I (RCA I), *Sambucus nigra* agglutinin (SNA), standard deviation (SD).

Introduction

Alzheimer's disease (AD) is a complex neurological disorder characterized by a progressive dementia. The amyloid hypothesis maintains that the pivotal event in AD is the production of amyloid-β (Aβ) peptides following the metabolism of the amyloid precursor protein (APP) [1]. Increasing concentrations of Aβ in the brain correlate with disease progression [2]. Critically, not all forms of Aβ have equal biological significance; toxicity is dependent upon the state of Aβ, whether that is the length of peptide, state of aggregation, homogeneity of aggregates or specific Aβ conformations. The key to understanding the amyloid hypothesis is the realization that there exist disease-relevant forms of Aβ, while other forms are less toxic or biologically inert [3] and may play a role in normal synapse function [4]. Thus, in this study we sought to identify factors involved in the production and release of toxic forms of Aβ. The pathogenesis of AD is intimately linked with the loss of synapse function [5, 6]. Many studies demonstrated close correlations between the loss of synaptic proteins such as synaptophysin, indicative of synapse degeneration, and the degree of dementia in AD [7, 8]. In this study the amounts of synaptophysin and cysteine string protein (CSP) were measured to quantify synapse density in cultured neurons incubated with Aβ. The loss of synaptic proteins from cultured neurons incubated with Aβ provides a useful *in vitro* model in which to investigate AD-related synapse damage.

7PA2 cells (Chinese hamster ovary cells stably transfected with human APP_{751}[9]) release the soluble Aβ oligomers that are considered to be key mediators of synapse damage in AD [10, 11]. The properties of soluble Aβ released from 7PA2 cells are similar to those derived from the brains of AD patients [12-14]. Since the production of Aβ is affected by the presence of the cellular prion protein (PrP^C) [15] the biochemistry of PrP^C-induced suppression of Aβ production in 7PA2 cells was examined. PrP^C is linked to membranes via a glycosylphosphatidylinositol (GPI) anchor [16] and is rapidly incorporated into living cells [17]. Here we show that treatment of 7PA2 cells with PrP^C reduced the release of toxic Aβ as measured by their ability to cause synapse degeneration in cultured neurons. This effect of PrP^C was dependent upon the composition of its GPI anchor; the GPI anchor attached to PrP^C is unusual in that it contains sialic acid [16] and PrP^C with a GPI anchor lacking sialic acid (desialylated PrP^C) did not alter the release of toxic Aβ. Further studies demonstrate that the suppression of toxic Aβ oligomers was achieved with specific sialylated GPIs alone. Treatment of 7PA2 cells with either PrP^C or sialylated GPIs reduced the release of Aβ oligomers but increased the release of Aβ monomers.

Methods

Culture of 7PA2 cells – Chinese hamster ovary (CHO) cells stably transfected with a cDNA encoding APP_{751} (7PA2 cells) were maintained as described [9]. For experiments 7PA2 cells were grown in 6 well plates until 80% confluent. Culture media was replaced with neurobasal medium containing B27 components (Invitrogen) ± test compounds and the cells cultured for a further 3 days and the conditioned medium (CM) from these cells (7PA2-CM) was collected. Cells were washed 3 times with ice cold PBS and homogenised in an extraction buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.2% SDS) containing mixed protease inhibitors (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, Aprotinin, Leupeptin, Bestatin, Pepstatin A and E-46) and a phosphatase inhibitor cocktail (PP1, PP2A, microcystin LR, cantharidin and p-bromotetramisole (Sigma)) at 10^6 cells/ml. Cellular debris was removed by centrifugation (20 minutes as 16000 x g and the supernatant collected. Both 7PA2-CM and cell extracts were centrifuged at 100,000 x *g* for 4 hours at 4^0C and passed through a 50 kDa filter (Sartorius). CM from CHO cells (CHO-CM) were used as controls. 7PA2-CM containing Aβ monomers were prepared by filtration through a 10 kDa filter (Sartorius). To deplete 7PA2-CM of Aβ they were incubated with 1 μg/ml mAb 4G8 (reactive with amino acids 17-24 of Aβ) or an isotype control (mock depletions) and incubated on rollers for 2 hours. Protein G microbeads were added (10 μl/ml) (Sigma) for 30 minutes and protein G bound-antibody-antigen complexes removed by centrifugation (16,000 x *g* for 5 minutes). For immunoblot analysis, 7PA2-CM/monomer or oligomer preparations were concentrated from 2000 to 100 μls using a 3 kDa filter (Sartorius). 10 μl of sample were mixed with an equal volume of in 0.5% NP-40, 5 mM CHAPS, 50 mM Tris, pH 7.4 and separated by electrophoresis. Proteins were transferred onto a Hybond-P polyvinylidene fluoride membrane by semi-dry blotting and blocked using 10% milk powder. Aβ was detected by incubation with mAb 6E10 (Covance), biotinylated anti-mouse IgG, extravidin-peroxidase and enhanced chemiluminescence.

Cell survival - To determine cell viability thiazolyl blue tetrazolium bromide was added to cells at a final concentration of 50 μM for 3 hours at 37ºC. 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. Neuronal survival was calculated with reference to untreated cells (100% survival).

Isolation of detergent-resistant membranes (DRMs) (lipid rafts)- These membranes were isolated by their insolubility in non-ionic detergents as described [18]. Briefly, cells were homogenised in an ice-cold buffer containing 1% Triton X-100, 10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 10 mM EDTA and mixed protease/phosphatase inhibitors and nuclei and large fragments were removed by centrifugation (300 x g

for 5 minutes at 4°C). The post nuclear supernatant was incubated on ice (4°C) for 1 hour and centrifuged (16,000 x g for 30 minutes at 4°C). The supernatant was reserved as the detergent soluble membrane (DSM) while the insoluble pellet was homogenised in extraction buffer supplemented with protease and phosphatase inhibitors (as above) at 10^6 cells/ml, centrifuged (10 minutes at 16,000 x g) and the soluble material was reserved as the DRM fraction.

Primary neuronal cultures - Cortical neurons were prepared from the brains of mouse embryos (day 15.5) after mechanical dissociation and cell sieving as described [19]. Neurons were plated at 2 x 10^5 cells/well in 48 well plates in Hams F12 containing 5% foetal calf serum for 2 hours. Cultures were shaken (600 r.p.m for 5 mins) and non-adherent cells removed by 2 washes in PBS. Cells were subsequently grown in neurobasal medium containing B27 components (Invitrogen) and nerve growth factor (5 ng/ml) (Sigma) for 10 days. Immunohistochemistry showed that greater than 90% of cells were neurofilament positive. Neurons were incubated with Aβ preparations or prostaglandin E_2 and synapse damage was assessed after 24 hours. Neurons were washed 3 times in PBS and homogenised in extraction buffer containing mixed protease and phosphatase inhibitors (as above) at 10^6 cells/ml. 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.

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 polyvinylidene 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), CSP with rabbit polyclonal anti-CSP ((sc-33154) Santa Cruz), vesicle-associated membrane protein (VAMP)-1 with mAb 4H302 (Abcam), rabbit polyclonal antibodies to caveolin (Upstate), platelet activating factor (PAF) receptor with rabbit polyclonal anti-PAF receptor (Cayman chemicals), APP with rabbit polyclonal anti-APP (Sigma) and PrP^C by mAb 4F2 (Jaques Grassi [20]). These were visualised using a combination of biotinylated anti-mouse/goat/rat/rabbit IgG (Sigma), extravidin-peroxidase and enhanced chemiluminescence.

Synaptophysin ELISA - Maxisorb immunoplates (Nunc) were coated with an anti-synaptophysin mouse mAb (MAB368 - Millipore) and bound synaptophysin was detected using rabbit polyclonal anti-synaptophysin (Abcam) followed by a biotinylated anti-rabbit IgG, extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenol phosphate solution (Sigma). Absorbance was measured on a microplate reader at 405 nm. Samples were expressed as "units synaptophysin" where 100 units were defined as the amount of synaptophysin in 10^6 control neurons.

CSP ELISA – Maxisorb immunoplates were coated with an anti-CSP mAb (Santa Cruz) and blocked with 5% milk powder. Samples were added and bound CSP was detected using rabbit polyclonal anti-CSP (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 were defined as the amount of CSP in 10^6 control neurons.

Isolation of PrP^C - PrP^C and the control Thy-1 protein were isolated from murine GT1 neuronal cell membranes using a combination of immunoaffinity columns, size exclusion chromatography (Superdex) and reverse-phase chromatography on C18 columns (Waters) as described [21]. N-linked glycans were removed from PrP^C by digestion with 2 units/ml endoglycosidase F (PNGase) (Sigma), monoacylated PrP^C by digestion with 100 units/ml bee venom phospholipase A_2 (PLA_2) (Sigma) and desialylated PrP^C by digestion with 0.2 units/ml neuraminidase (*Clostridium perfringens* - Sigma) for 2 hours at 37°C [21]. Digested PrP^C preparations were purified using reverse phase chromatography (C18 columns); PrP positive fractions were pooled, desalted and lyophilised. For tissue culture studies PrP-containing fractions were solubilised in culture medium by sonication prior to further use.

PrP ELISA - The amount of PrP in samples was measured by ELISA as described [21]. Briefly, maxisorb immunoplates were coated with mAb ICSM18 (gift Dr M Tayebi, Royal Veterinary College) and blocked with 5% milk powder. Samples were applied and detected with biotinylated mAb ICSM35 (gift Dr M Tayebi), followed by extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenyl phosphate solution. Absorbance was measured at 405 nm and the concentrations of PrP in samples were calculated by reference to serial dilutions of recombinant murine PrP (Prionics).

Isolation of GPIs - Purified proteins were digested with 100 μg/ml proteinase K, at 37°C for 24 hours, resulting in GPIs attached to the terminal amino acid. The released GPIs were extracted with water-saturated butanol, washed with water 5 times and loaded onto C18 columns. GPIs were eluted under a gradient of propanol and water. The presence of GPIs was detected by ELISA as described [21]. Maxisorb immunoplates were coated with 0.5 μg/ml concanavalin A (binds mannose) and blocked with 5% milk powder in PBS-tween. Samples were added and any bound GPI was detected by the addition of the phosphatidylinositol-reactive mAb 5AB3-11, followed by a biotinylated anti-mouse Ig (Sigma), extravidin-alkaline phosphatase and 1mg/ml 4-nitrophenyl phosphate solution. Absorbance was measured on a microplate reader at 405 nm. For some experiments GPIs derived from PrP^C were digested with 100 units/ml bee venom PLA_2 (monoacylated GPI) or 0.2 units/ml neuraminidase (desialylated GPI) for 2 hours

at 37°C. The modified GPIs were isolated on C18 columns as above. GPIs were dissolved in ethanol at 2 μM (stock solutions) and diluted in tissue culture medium for bioassays.

Analysis of GPIs - The presence of phosphatidylinositol in GPI anchors was identified using mAb (5AB3-11) and specific glycans were detected with biotinylated lectins. Isolated GPIs were bound to nitrocellulose membranes by dot blot and blocked with 5% milk powder. Samples were incubated with mAb 5AB3-11, biotinylated *Sambucus nigra* agglutinin (SNA) (detects terminal sialic acid residues bound α-2,6 or α-2,3 to galactose), biotinylated concanavalin A (detects mannose) or biotinylated *Ricinus Communis* Agglutinin I (RCA-I) (detects terminal galactose) (Vector Labs). Bound lectins were visualised using extravidin peroxidase and enhanced chemiluminescence. The mAb was visualised by incubation with a horseradish peroxidase conjugated anti-murine-Ig and chemiluminescence. GPIs were separated by high performance thin layer chromatography (HPTLC) on silica gel 60 plates using a mixture of chloroform/methanol/water (10/10/3 v/v/v). Plates were soaked in 0.1 % poly isobutyl methacrylate in hexane, dried, and blocked with 5% milk powder. GPIs were detected with a mAb that binds to phosphatidylinositol as described [22].

Brain extracts – Soluble extracts were prepared from brain tissue from patients with a clinical, and pathologically-confirmed, diagnosis of Alzheimer's disease as described [12]. Briefly, brain tissue, stored at -80°C was thawed, cut into pieces of approximately 100 mg and 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 so that there was the equivalent of 100 mg brain tissue/ml. The 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 plaque Aβ. The soluble material was then desalted (3 kDa filter (Sartorius)) to eliminate bioactive small molecules and drugs and the retained material collected (preparation contains peptides with molecular weights between 3 and 50 kDa) and stored at -80°C.

APP ELISA – Maxisorb immunoplates were coated with mouse mAb anti-APP (Clone 1G6) Biolegend (epitope 573-596)) and blocked with 5% milk powder in PBS-tween and samples were applied for 1 hour. Bound APP was detected using rabbit polyclonal antibodies against the N-terminal of APP (amino acids 40 to 60 - Sigma) followed by anti-rabbit IgG conjugated to alkaline phosphatase followed by 1 mg/ml 4-nitrophenol phosphate solution and optical density was read in a spectrophotometer at 405 nm. Results were calculated by comparison to serial dilutions of cell extracts from control cells.

Sample preparation – To detach $A\beta_{42}$ from membrane components that block 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, dried, suspended in a buffer containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA and 0.2% SDS and sonicated.

$A\beta_{42}$ ELISA –Nunc Maxisorb immunoplates were coated with mAb 4G8 (epitope 17-24) (Covance) in carbonate buffer overnight. Plates were blocked with 5% milk powder in PBS-tween and samples were applied. The detection antibody was an $A\beta_{42}$ selective rabbit mAb BA3-9 (Covance) followed by biotinylated anti-rabbit IgG and extravidin alkaline phosphatase (Sigma). Total Aβ was visualised by addition of 1 ng/ml 4-nitrophenol phosphate solution and optical density was read in a spectrophotometer at 405 nm.

$A\beta_{40}$ ELISA - Maxisorb immunoplates were coated with mAb 4G8 (epitope 17-24) and blocked with 5% milk powder in PBS-tween. Samples were applied and $A\beta_{40}$ was detected with rabbit polyclonal PC-149 (Merck) followed by biotinylated anti-rabbit IgG and extravidin alkaline phosphatase. Total Aβ was visualised by addition of 1 ng/ml 4-nitrophenol phosphate and optical density was read in a spectrophotometer at 405 nm.

Statistical Analysis - Comparison of treatment effects was carried out using Student's paired t-tests. Error values are standard deviation (SD) and significance was determined where $P<0.01$. Bivariate analysis using Pearson's coefficient (IBM SPSS statistics 20) were used to examine correlations between data sets.

Results

7PA2 cells release toxic Aβ – In support of the hypothesis that Aβ caused synapse damage, the CM from 7PA2 cells caused dose-dependent reductions in synapsin-1, VAMP-1, CSP and synaptophysin from cultured neurons indicative of synapse damage (Figure 1A). The addition of 100 µg/ml 7PA2-CM did not reduce neuronal viability as measured by thiazolyl blue tetrazolium ((101% cell survival ± 6 compared with 100% ± 5, n=9, P=0.6) indicating that synapse degeneration occurred in the absence of any significant neuronal death. Whereas 7PA2-CM caused dose-dependent reductions in both synaptophysin (Figure 1B) and CSP (Figure 1C), the addition of Aβ-depleted CM did not, indicating that Aβ was the synaptotoxic element. Immunoprecipitation studies demonstrated that the toxic entity in these CM was Aβ. Immunodepletion with mAb G48 reduced the concentrations of both $A\beta_{42}$ (0.04 nM $A\beta_{42}$ ± 0.04 compared with 2.21 nM ± 0.3, n=9, $P<0.01$) and $A\beta_{40}$ (0.57 nM $A\beta_{40}$ ± 0.28 nM compared with 7.42 nM $A\beta_{40}$ ± 0.6

nM $A\beta_{40}$, n=9, P=0.38). Mock-depletions with a control IgG mAb did not significantly affect the concentration of either $A\beta_{40}$ (6.95 nm $A\beta_{40}$ ± 0.81 compared with 7.42 nM $A\beta_{40}$ ± 0.6, n=9, P=0.2) or $A\beta_{42}$ (2.03 nM $A\beta_{42}$ ± 0.21 compared with 2.21 $A\beta_{42}$ ± 0.3 nM, n=9, P=0.21). Not all forms of Aβ have equal biological significance, as toxicity is dependent upon the state of Aβ. To determine whether 7PA2-CM contained Aβ similar to the toxic Aβ in the brains of Alzheimer's patients it was compared to that of soluble brain extracts. 7PA2-CM and brain extracts containing similar concentrations of $A\beta_{42}$ caused similar dose-dependent reductions in synaptophysin (Figure 1D) and CSP (Figure 1E).

PrP^C reduced the release of toxic Aβ from 7PA2 cells – Since PrP^C had been reported to inhibit the production of $A\beta_{42}$ [15] the effects of PrP^C on 7PA2 cells were studied. The viability of 7PA2 cells, as measured by thiazolyl blue tetrazolium, was not significantly affected by 10 nM PrP^C (102% cell survival ± 6 compared with 100% ± 5, n=6, P=0.37). CM from 7PA2 cells treated with 10 nM PrP^C did not cause synapse damage, as measured by the loss of synaptophysin (Figure 2A) from cultured neurons. 10 nM PrP^C was used in these assays as this is the PrP^C concentration in neurons [23]. The effects of PrP^C were dose-dependent. CM from cells with recombinant PrP (PrP protein lacking any post-translational modifications) had similar effects to CM from control cells indicating that the inhibitory effect of PrP^C was due to a post-translational modification. The effects of N-linked glycans PrP^C upon the production of toxic Aβ were examined; CM from 7PA2 cells treated with PrP^C from which N-linked glycans had been removed did not cause synapse damage. There were no significant differences between PrP^C and PrP^C lacking N-linked glycans upon the suppression of toxic Aβ production as measured by loss of synaptophysin (Figure 2B).

PrP^C-mediated suppression of toxic Aβ release is dependent upon its GPI - PrP^C is connected to cell membranes by a GPI anchor [16]. The role of the GPI in PrP^C-mediated suppression of toxic Aβ was examined using monoacylated PrP^C, a form of PrP^C differing from PrP^C only in the lack of an acyl chain in its GPI anchor [24]. Following the addition of 10 nM PrP^C preparations similar amounts of PrP^C and monoacylated PrP^C were found in 7PA2 cells (9.3 nM ± 0.6 compared with 9.5 nM ± 0.5, P=0.74, n=9). In addition the viability of 7PA2 cells as measured by thiazolyl blue tetrazolium was not significantly affected by 10 nM monoacylated PrP^C (101% cell survival ± 6 compared with 100% ± 5, n=6, P=0.75). CM from cells treated with 10 nM monoacylated PrP^C caused a reduction in synaptophysin similar to CM from untreated cells demonstrating that the inhibitory effect of PrP^C on toxic Aβ production was GPI-dependent (Figure 2C). The CM from 7PA2 cells treated with Thy-1, another GPI-anchored protein, caused synapse damage, as measured by the loss of synaptophysin, similar to CM from control cells (Figure 2C) indicating that not all GPI-anchored proteins had this suppressive affect. Since the toxicity of 7PA2-CM is Aβ-dependent it was surprising to see that treatment with 10 nM PrP^C had only a small effect on the

concentrations of $A\beta_{42}$ (2.05 nM ± 0.22 compared with 2.45 nM ± 0.38, n=9, P=0.02) or $A\beta_{40}$ (6.94 nM ± 1.04 compared with 7.28 nM ± 0.72, n=9, P=0.24) in CM.

PrP^C reduced the release of Aβ oligomers – Reports that Aβ monomers are not toxic [25, 26] suggested that the toxicity of 7PA2-CM was relative to the concentrations of Aβ oligomers rather than the total Aβ. Immunoblots showed that 7PA2-CM contained monomers, dimers and trimers (Figure 3A, Lane 1) while 7PA2-CM retained by a 10 kDa filter was depleted of monomers but contained dimers and trimers (Figure 3A, Lane 2). Treatment with PrP^C, but not monoacylated PrP^C, caused a dose-dependent reduction in $A\beta_{42}$ oligomers (Figure 3B). Treatment with 10 nM PrP^C also reduced the concentrations of $A\beta_{40}$ oligomers from 0.41 nM ± 0.16 to 0.2 nM ± 0.14, n=9, P=0.03 whereas 10 nM monoacylated PrP^C had no significant effect (0.47 nM ± 0.11 compared with 0.41 ± 0.16, n=9, P=0.4). There was a significant inverse correlation between the concentrations $A\beta_{42}$ oligomers in CM from cells treated with PrP^C (1.25 to 10 nM) and amounts of synaptophysin in neurons incubated with these CM, Pearson's coefficient= -0.91, P<0.01 (Figure 3C).

Immunoblots showed that 7PA2-CM that passed through a 10 kDa filter contained only Aβ monomers (Figure 4A, Lane 2). Treatment with PrP^C, but not monoacylated PrP^C caused a dose-dependent increase in the concentrations of $A\beta_{42}$ monomers in CM (Figure 4B). Treatment with 10 nM PrP^C did not significantly alter the concentrations of $A\beta_{40}$ monomers (7.1 nM $A\beta_{40}$ ± 0.62 compared with 6.6 nM ± 0.58, n=9, P=0.07). A significant inverse correlation between the concentrations of $A\beta_{42}$ oligomers and $A\beta_{42}$ monomers in CM from 7PA2 cells incubated with PrP^C (1.25 to 10 nM), Pearson's coefficient= -0.75, P<0.01 was observed (Figure 4C).

GPIs reduced the release of toxic Aβ – Since these results indicated that the GPI attached to PrP^C had a role in suppressing $A\beta_{42}$ oligomer production, we hypothesised that GPIs alone could alter Aβ production. Analysis of GPIs isolated from PrP^C and Thy-1 by HPTLC (Figure 5A) and reverse phase chromatography on C18 columns (Figure 5B) showed that they had different properties. Whereas CM from 7PA2 cells treated with 10 nM GPIs derived from PrP^C did not cause synapse damage (did not affect the synaptophysin (Figure 5C) or CSP (Figure 5D) content of neurons), the CM from 7PA2 cells treated with 10 nM GPIs isolated from Thy-1 caused extensive loss of synaptophysin and CSP. The effects of PrP^C-derived GPIs on the production of toxic Aβ were dose-dependent (Figure 5E). These results showed that the GPI-induced suppression of toxic Aβ production was structure dependent.

CM from cells treated with PrP^C-derived GPIs, but not Thy-1-derived GPIs, showed a dose-dependent reduction in $A\beta_{42}$ oligomers (Figure 6A) and increase in $A\beta_{42}$ monomers (Figure 6B). There was a

significant inverse correlation between concentrations of $A\beta_{42}$ oligomers and $A\beta_{42}$ monomers in CM from 7PA2 cells incubated with PrP^C-derived GPIs (1.25 to 10 nM), Pearson's coefficient= 0.87, $P<0.01$ (Figure 6C). The loss of Aβ oligomers and corresponding increase in Aβ monomers suggested that the GPIs may interact directly with Aβ oligomers causing them to dissociate into monomers. When Aβ preparations (containing 10 nM $A\beta_{42}$) and incubated with 10 nM GPIs at 37°C for 3 days were analysed by electrophoresis and immunoblot there was no difference in the proportion of oligomers and monomers (Figure 6D). In addition, monomeric forms of $A\beta_{42}$ were not found in Aβ oligomer preparations incubated with GPIs; indicating that GPIs did not cause the dissociation of Aβ oligomers.

Sialylated GPIs reduced the release of toxic $A\beta_{42}$ – The composition of GPIs derived from PrP^C are unusual in that they contain sialic acid [16] and (Figure 7A), a rare modification of mammalian GPIs. To examine the structure-function relationship in more detail the GPIs derived from PrP^C were digested to create desialylated GPIs and monoacylated GPIs. GPIs, desialylated GPIs and monoacylated GPIs were visualised by HPTLC (Figure 7B) and isolated by reverse phase chromatography on C18 columns (Figure 7C). In dot blots all GPIs reacted with mAb 5AB3-11 (reactive with phosphatidylinositol) and concanavalin A (mannose). SNA (detects terminal sialic acid residues bound α-2,6 or α-2,3 to galactose) bound to GPIs and monoacylated GPIs but not to desialylated GPIs (Figure 7D). Conversely, RCA-1 (detects terminal galactose) only bound to desialylated GPIs.

The CM from 7PA2 cells treated with 10 nM GPI preparations was added to cultured neurons. CM from 7PA2 cells treated with control medium, 10 nM monoacylated GPIs or 10 nM desialylated GPIs reduced the amounts of synaptophysin in neurons, whereas CM from cells treated with GPIs did not (Figure 8A). The effects of PrP^C-derived GPIs were dose-dependent (Figure 8B). Treatment of 7PA2 cells with 10 nM GPIs, but not monoacylated GPIs or desialylated GPIs (10 nM), reduced the concentrations of $A\beta_{42}$ oligomers (Figure 8C) and increased the concentrations of $A\beta_{42}$ monomers in CM (Figure 8D).

GPIs increased the release of neuroprotective Aβ – To determine if Aβ monomers have a protective role the CM from GPI-treated 7PA2 cells were mixed with brain extract containing 2 nM $A\beta_{42}$ and incubated with neurons. The brain extract caused a reduction in synaptophysin (Figure 9A) and CSP (Figure 9B). The presence of CM from GPI-treated 7PA2 cells protected neurons against the brain extract (Aβ)-induced synapse damage. It was important to determine whether the protective effect of CM from treated 7PA2 cells was mediated by Aβ. Therefore, brain extracts were incubated with CM from GPI-treated cells after Aβ had been removed by immunoprecipitation and added to neurons. Removal of Aβ removed the protective effects of CM from GPI-treated cells (Figures 9A & B). Next, Aβ monomers were isolated from the CM of

GPI-treated cells. Aβ monomers blocked the brain extract-induced reductions in synaptophysin (Figure 9C) and CSP (Figure 9D). The protective effects of monomer preparations derived from GPI-treated 7PA2 cells were compared to that of monomers prepared from brain extracts. Monomer preparations derived from 7PA2-CM and brain extract had similar activity when based on their $A\beta_{42}$ content (Figure 9E). The protective effect of these monomer preparations was stimulus specific; there were no significant differences in either synaptophysin (31 units ± 7 compared with 34 ± 10, n=9, P=0.37) or CSP (54 units ± 6 compared with 50 ± 13, n=9, P=0.4) content of neurons incubated with 10 nM prostaglandin E_2 ± monomer preparations containing 10 nM $A\beta_{42}$.

PrP^C and GPIs reduced cell-associated Aβ - The possibility that PrP^C or GPIs affected the release, rather than the production of Aβ was examined by measuring Aβ in cell extracts from treated 7PA2 cells. The concentrations of $A\beta_{42}$ in 7PA2 cells was significantly reduced by treatment with 10 nM PrP^C (6.7 nM $A\beta_{42}$ ± 1.7 compared with 12.15 nM ± 1.93, n=12, P<0.01) or with 10 nM GPIs derived from PrP^C (5.67 nM $A\beta_{42}$ ± 1.34 compared with 12.15 nM ± 1.93, n=12, P<0.01). The concentrations of $A\beta_{42}$ were not significantly affected by treatment with 10 nM monoacylated PrP^C (12.55 nM $A\beta_{42}$ ± 2.1 compared with 12.15 nM ± 1.93, *n=12*, P=0.74) or 10 nM desialylated GPIs (12.76 nM $A\beta_{42}$ ± 2.16 compared with 12.15 nM ± 1.93, n=12, P=0.42).

GPIs altered the distribution of APP – Further studies examined the effects of GPIs upon APP. Treatment with 10 nM GPI did not significantly alter concentrations of APP within cells (97 units ± 3.4 compared with 100 units ± 2.8, n=6, P=0.14). The processing of APP to toxic Aβ peptides is thought to occur within membrane micro-domains called lipid rafts and in control cells approximately 25% of cellular APP was found within DRMs (lipid rafts). Treatment with 10 nM GPI, but not 10 nM desialylated GPI, reduced the amounts of APP found within DRMs in a dose-dependent manner (Figure 10A). Treatment of 7PA2 cells with 10 nM GPI did not affect the amounts of other raft-associated proteins including caveolin and the PAF receptor in DRMs (Figure 10B). In cells treated with GPIs (1.25 to 10 nM) there was a significant correlation between the amounts of APP found in lipid rafts and the concentrations of $A\beta_{42}$ oligomers found in CM (Figure 10C). There was also a significant inverse correlation between the amounts of APP found in lipid rafts and the concentrations of $A\beta_{42}$ monomers found in CM (Figure 10D).

Discussion

There are 2 key findings from this study; firstly that the release of toxic Aβ from 7PA2 cells is controlled by a pathway sensitive to the presence of PrP^C. More specifically that it was the sialic acid contained within the GPI anchor attached to PrP^C that affected Aβ production. Secondly, we report that the GPI-induced changes involved both a reduction in toxic Aβ oligomers and an increase in neuroprotective Aβ monomers.

This study concentrated on the biologically active forms of Aβ released by 7PA2 cells by measuring their effects upon synapses (based on reports that synapse damage, as measured by the loss of synaptic proteins, is a good correlate of dementia in AD [7, 8, 27, 28]). The finding that synapse damage caused by CM from 7PA2 cells was comparable to that caused by soluble Alzheimer's brain extracts (with regard to their $A\beta_{42}$ content) implied that 7PA2 cells release toxic Aβ oligomers that are similar to those found within the brains of AD patients. The major observation was that the CM collected from 7PA2 cells treated with PrP^C did not damage synapses. However, in contrast to the initial report that expression of PrP^C reduced Aβ production in transfected human neuroblastoma cells [15] we found that PrP^C had only minor effects on the concentrations of $A\beta_{40}$ and $A\beta_{42}$. It should be noted that the 2 systems are very different with regard to cell types and the amounts of PrP^C expressed.

Observations that recombinant PrP, which does not contain post-translational modifications such as GPIs and N-linked glycans, did not affect the production of toxic Aβ demonstrated that the protein alone was not responsible for suppression of Aβ production and led us to examine the effects of post-translational modifications of PrP^C. We concluded that N-linked glycans were not necessary for the suppression of toxic Aβ production as their removal from PrP^C also inhibited the production of toxic Aβ. In contrast, monoacylated PrP^C, a form of PrP^C that differed only in the composition of its GPI anchor, did not suppress toxic Aβ release indicating a role for the GPI anchor in regulating Aβ production. The observation that isolated GPIs were capable of suppressing toxic Aβ release and altering the ratio of Aβ monomers to Aβ oligomers showed that the GPI alone affected Aβ production and that the GPI was not simply a contributory factor to protein interactions mediated by PrP^C. The structure of the GPI was important as GPIs isolated from Thy-1, or GPIs derived from PrP^C that had been modified (monoacylated or desialylated GPIs) did not affect the release of toxic Aβ.

The observation that PrP^C reduced the toxicity of CM from 7PA2 cells without causing major changes in the concentrations of $A\beta_{40}$ and $A\beta_{42}$ indicated that it affected the forms of Aβ produced. Thus, the presence of PrP^C has 2 major effects; it reduced the concentrations of $A\beta_{42}$ oligomers, responsible for synapse

damage, and it increased the release of $A\beta_{42}$ monomers. Immunodepletion and filtration studies showed that the protective effect was mediated by Aβ monomers, an observation consistent with a prior report [29]. These results are consistent with the hypothesis that it is the ratio of Aβ monomers to Aβ oligomers that is critical factor in determining the toxicity of CM and explained the observation that CM from cells treated with GPIs (which increased concentrations of $A\beta_{42}$ monomers) blocked synapse damage caused by soluble Aβ oligomers. The protective effects of Aβ monomers were stimulus specific; they did not affect synapse damage induced by prostaglandin E_2 indicating a selective rather than a universal action.

The GPIs did not have a direct effect on $A\beta_{42}$ oligomers (did not cause the dissociation of oligomers into monomers); rather the effects of GPIs were upon the production of oligomers and monomers. Currently we can only speculate how specific sialylated GPIs might alter the production of toxic Aβ. Observations that GPIs help solubilise cholesterol and that GPI-anchored proteins triggered the formation of lipid rafts [30, 31] implicate GPI-anchored proteins as regulators of lipid raft structure and function. The processing of APP by β- and γ-secretases to form Aβ is affected by the composition of cell membranes; more specifically the form and function of lipid rafts [32, 33]. Notably, PrP^C-mediated inhibition of β-secretase was dependent upon lipid rafts [15] and in this study monoacylated PrP^C and monoacylated GPIs, which were not found within lipid rafts [24], did not affect Aβ production, observations that support the idea that GPIs affected lipid raft function.

Not all GPIs suppressed the production of toxic Aβ; GPIs derived from Thy-1, monoacylated PrP^C or desialylated PrP^C had an inhibitory effect. The glycan structure of the GPI anchor mediates protein association with specific rafts [34] and affects the function of those rafts [35]. The composition of lipid rafts surrounding GPIs are dependent upon multiple interactions between the glycans and membrane lipids [36, 37] and the removal of sialic acid from the GPI of PrP^C changed the properties of the surrounding lipid raft; it allowed increased concentrations of gangliosides and cholesterol [22]. Although APP metabolism to toxic Aβ is thought to occur within lipid rafts [38] cells contain multiple, heterogeneous lipid rafts each with different composition and functions [39]. We hypothesised that GPIs derived from PrP^C are targeted to lipid rafts involved in the metabolism of APP to toxic Aβ. Lipid rafts are enriched with signalling molecules and act as domains in which the GPI anchors attached to PrP^C interact with cell signalling pathways [40]. The GPIs attached to PrP^C activate $cPLA_2$ [22] an enzyme that affects APP processing [41]. As this enzyme is essential for the maintenance of the endoplasmic reticulum-trans golgi network [42], a pathway reported to regulate APP metabolism [43], then inhibition of $cPLA_2$ may affect the intracellular trafficking of APP and hence its metabolism to toxic Aβ.

APP and many of the enzymes involved in the generation of Aβ are found in lipid rafts [44-46] and in control 7PA2 cells approximately 25% of APP was found within lipid rafts. The observation that GPIs reduced the amounts of APP within lipid rafts is consistent with reports that GPIs sequester cholesterol and consequently affect lipid raft composition and function [31, 47]. The finding that there was a significant positive correlation between the concentrations of $A\beta_{42}$ oligomers and the amounts of APP in lipid rafts of $A\beta_{42}$ monomers suggests that membrane targeting of APP is a key factor in production of Aβ oligomers. The protein cargos of the cell membrane traffic via different pathways to those within lipid rafts [48] and APP in lipid rafts may be targeted to different cell compartments (and consequently interacts with a different range of enzymes) than APP found in the cell membrane.

In summary this study demonstrated that the release of toxic Aβ by 7PA2 cells is sensitive to the presence of PrP^C and more specifically, its sialylated GPI anchor. Critically, sialylated GPIs derived from PrP^C increased the release of neuroprotective Aβ monomers and reduced the release of toxic Aβ oligomers.

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Figures

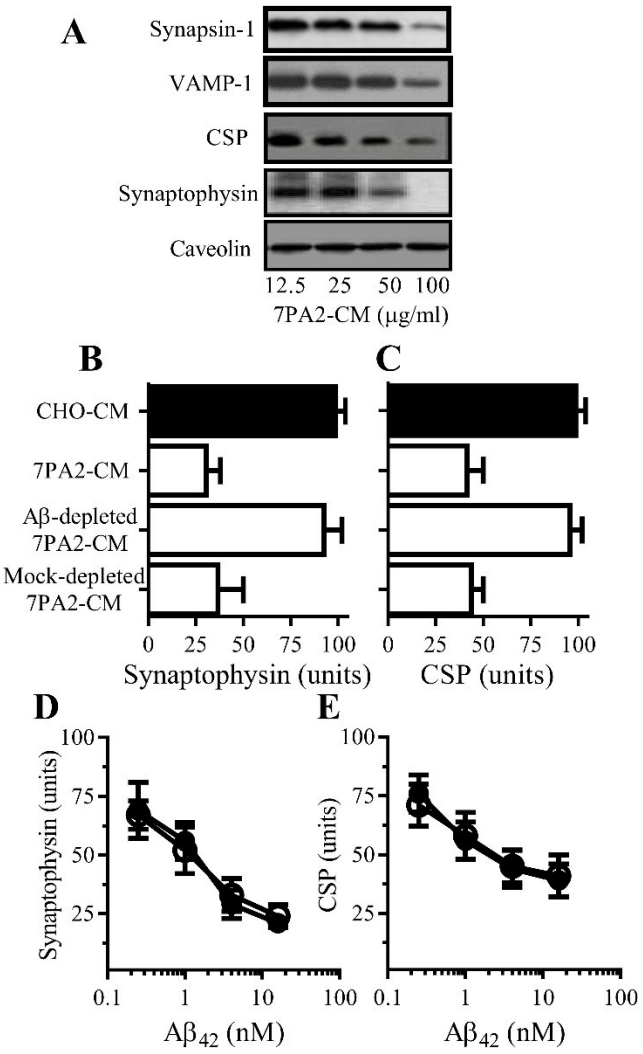

Figure 1. Soluble Aβ caused synapse damage in neurons - (A) Immunoblots showing the amounts of synapsin-1, VAMP-1, CSP, synaptophysin and caveolin in neurons incubated with 7PA2-CM as shown. The amounts of synaptophysin (B) and CSP (C) in neurons incubated with CHO-CM (■) or 7PA2-CM, Aβ-depleted 7PA2-CM or mock-depleted 7PA2-CM (□). Values are means ± SD from triplicate experiments performed 3 times, *n*=9. The amounts of synaptophysin (D) and CSP (E) in neurons incubated with 7PA2-CM (○) or soluble brain extract (●) containing $A\beta_{42}$ as shown. Values are means ± SD from triplicate experiments performed 3 times, *n*=9.

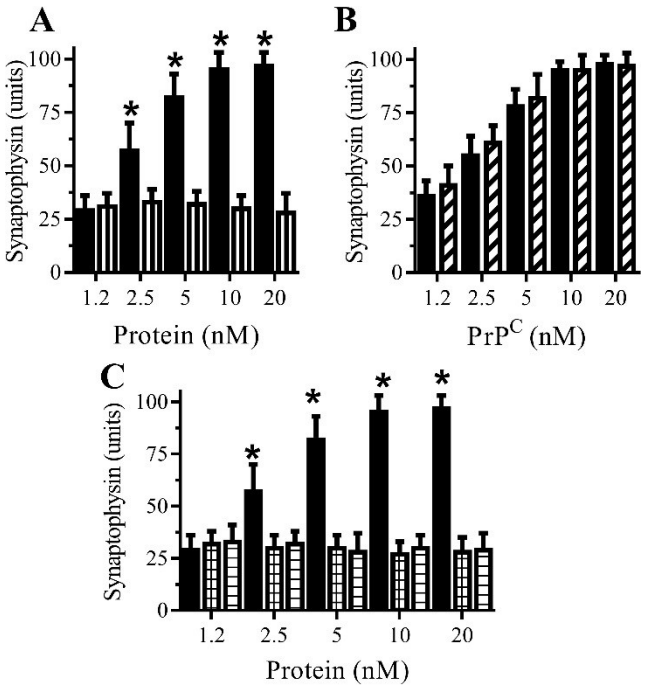

Figure 2. PrP^C reduced the release of toxic Aβ – (A) The amounts of synaptophysin in neurons incubated with CM from 7PA2 cells treated with concentrations of PrP^C (■) or recombinant PrP (vertical striped bars) as shown. Values are means ± SD from triplicate experiments performed 3 times, *n*=9. *=amounts of synaptophysin significantly greater than in neurons incubated with CM from untreated cells, P<0.05. (B) The amounts of synaptophysin in neurons incubated with CM from 7PA2 cells treated with concentrations of PrP^C (■) or PrP^C without N-linked glycans (striped bars) as shown. Values are means ± SD from triplicate experiments performed 3 times, *n*=9. (C) The amounts of synaptophysin in neurons incubated with CM from 7PA2 cells treated with concentrations of PrP^C (■), Thy-1 (hatched bars) or monoacylated PrP^C (horizontal striped bars) as shown. Values are means ± SD from triplicate experiments performed 3 times, *n*=9. *=amounts of synaptophysin significantly greater than in neurons incubated with CM from untreated cells, P<0.05.

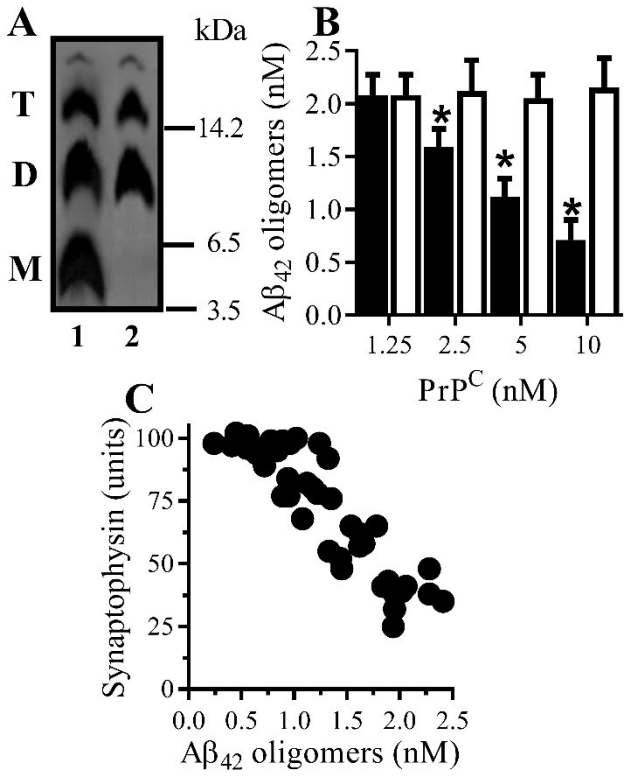

Figure 3. PrP^C reduced the release of Aβ oligomers (A) Immunoblot showing forms of Aβ (monomers (M), dimers (D) and trimers (T)) in 7PA2-CM (1) and 7PA2-CM retained by a 10 kDa filter (2). (B) The concentrations of $A\beta_{42}$ oligomers in CM from 7PA2 cells treated with PrP^C (■) or monoacylated PrP^C (□) as shown. Values are means ± SD from triplicate experiments performed 3 times, *n*=9. *= concentrations of Aβ significantly less than in control 7PA2-CM, P<0.05 (C) There was a significant inverse correlation between the concentrations of $A\beta_{42}$ oligomers in CM from cells treated with PrP^C (10 to 1.25 nM) and the amounts of synaptophysin in neurons incubated with these CM, Pearson's coefficient= -0.91, *P*<0.01.

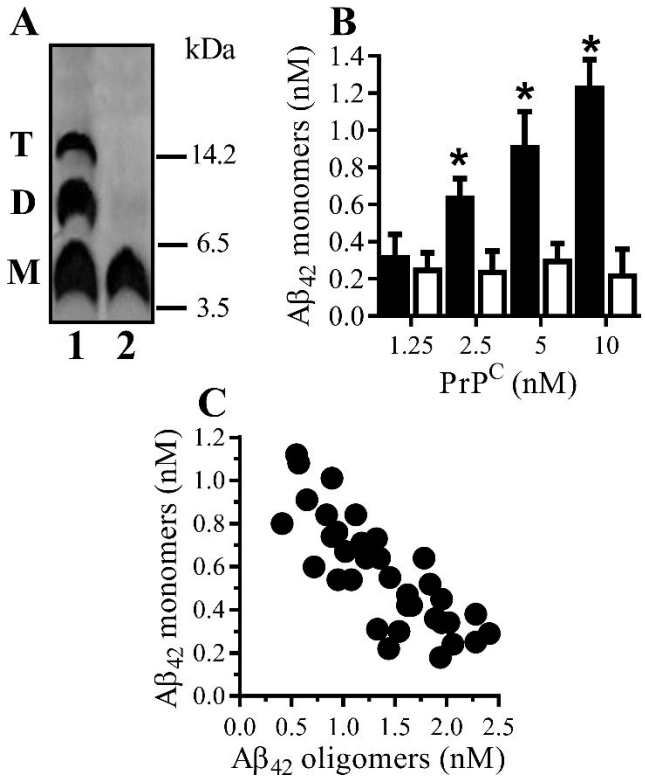

Figure 4. PrP^C increased the release of Aβ monomers – (A) Immunoblot showing forms of Aβ (monomers (M), dimers (D) and trimers (T)) in 7PA2-CM (1) and in 7PA2-CM that passed through a 10 kDa filter (2). (B) The concentrations of $A\beta_{42}$ monomers in CM from 7PA2 cells treated with PrP^C (■) or monoacylated PrP^C (□) as shown. Values are means ± SD from triplicate experiments performed 3 times, *n*=9. *=concentration of $A\beta_{42}$ monomers significantly higher than in control 7PA2-CM, P<0.05. (C) There was a significant inverse correlation between concentrations of $A\beta_{42}$ oligomers and $A\beta_{42}$ monomers in CM from 7PA2 cells incubated with PrP^C (10 to 1.25 nM), Pearson's coefficient= -0.75, *P*<0.01.

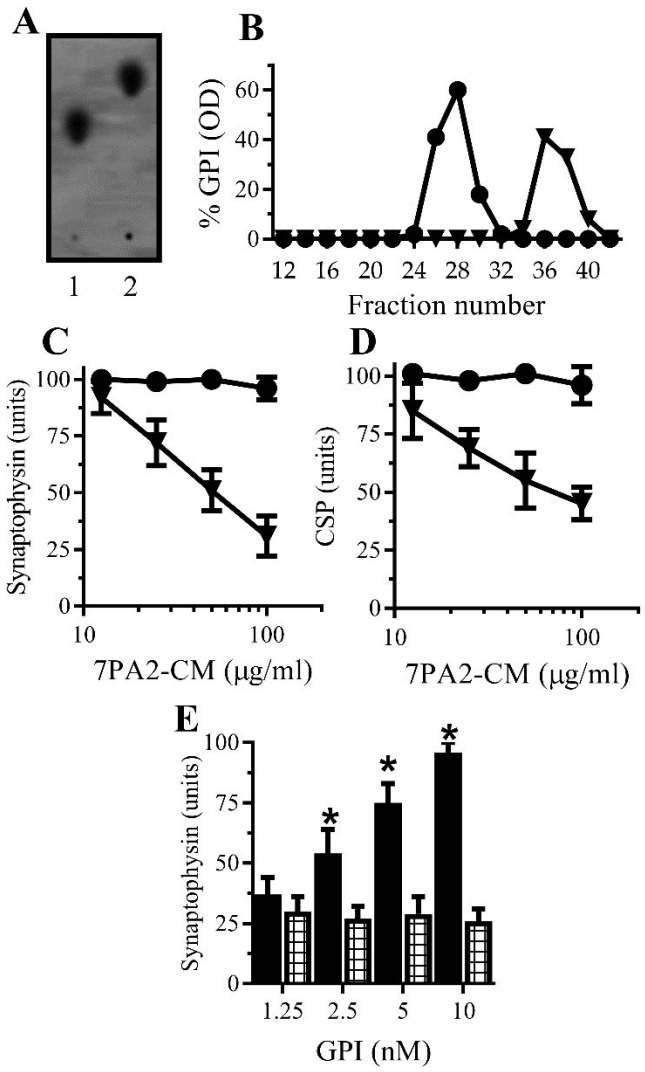

Figure 5. GPIs reduced the release of toxic Aβ – (A) Blot showing GPIs derived from PrP^C (1) or Thy-1 (2) separated by HPTLC. (B) The amounts of GPIs derived from PrP^C (●) or Thy-1 (▼) in fractions eluted from a C18 column. Values are means of duplicates. The amounts of synaptophysin (C) and CSP (D) in neurons incubated with CM from 7PA2 cells treated with 10 nM GPIs derived from PrP^C (●) or from Thy-1 (▼).Values are means ± SD from triplicate experiments performed 3 times, *n*=9. (E) The amounts of synaptophysin in neurons incubated with CM from 7PA2 cells treated with GPIs derived from PrP^C (■) or Thy-1 (hatched bars) as shown. Values are means ± SD from triplicate experiments performed 3 times, *n*=9. *=amounts of synaptophysin significantly greater than in neurons incubated with 7PA2-CM, $P<0.05$.

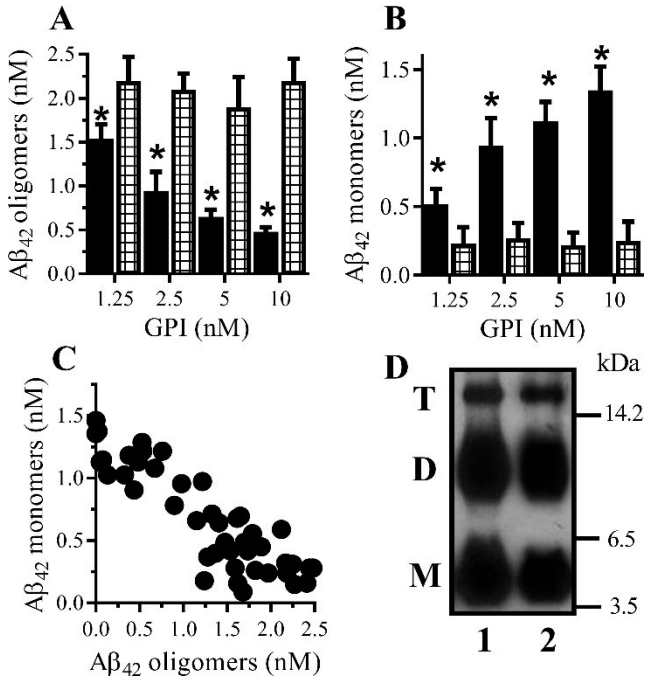

Figure 6. GPIs increased the release of Aβ monomers - The concentrations of $A\beta_{42}$ oligomers (A) and $A\beta_{42}$ monomers (B) in CM from 7PA2 cells treated with GPIs derived from PrP^C (■) or Thy-1 (hatched bars) as shown. Values are means ± SD from triplicate experiments performed 3 times, *n*=9. *=concentrations of $A\beta_{42}$ oligomers/monomers significantly different from those in CM from control cells, *P*<0.05. (C) There was a significant inverse correlation between concentrations of $A\beta_{42}$ oligomers and $A\beta_{42}$ monomers in CM from 7PA2 cells treated with PrP^C-derived GPIs (10 to 1.25 nM), Pearson's coefficient= 0.87, *P*<0.01. (D) Immunoblot showing forms of Aβ (monomers (M), dimers (D) and trimers (T)) in 7PA2-CM incubated at 37ºC for 3 days with control medium (1) or 10 nM GPIs (2).

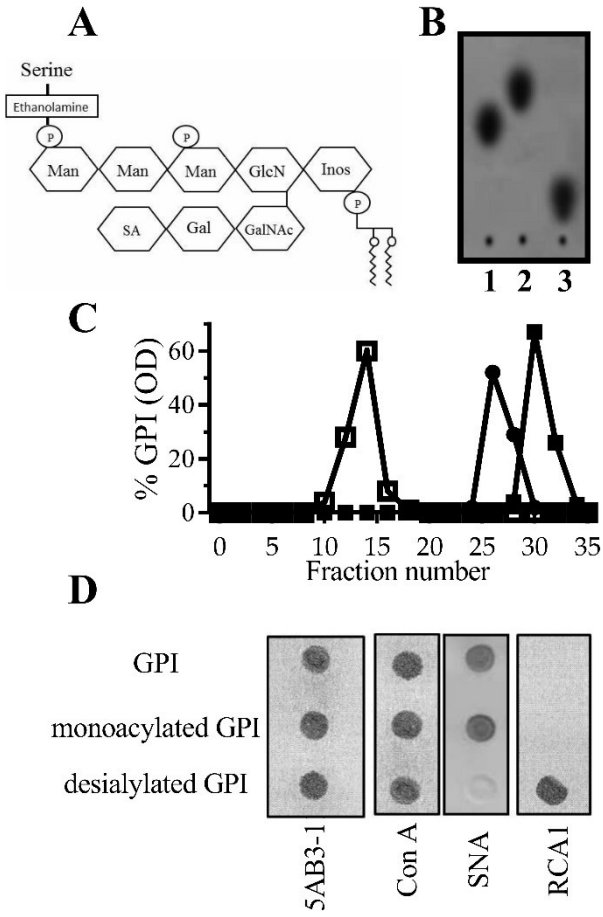

Figure 7. Analysis of GPIs - (A) Schematic showing the putative GPI derived from PrP^C. (B) Blot showing GPIs (1), desialylated GPIs (2) and monoacylated GPIs (3) separated by HPTLC. (C) The amounts of GPIs in fractions eluted from a C18 column loaded with GPIs derived from PrP^C (●), monoacylated GPIs (□) and desialylated GPIs (■). Values are means of duplicates. (D) Dot blots showing the reactivity of mAb 5AB3-11 (reactive with phosphatidylinositol), biotinylated concanavalin A (reacts with mannose), biotinylated SNA (reactive with terminal sialic acid) or biotinylated RCA-1 (reactive with terminal galactose) with GPIs derived from PrP^C, monoacylated-GPIs and desialylated GPIs.

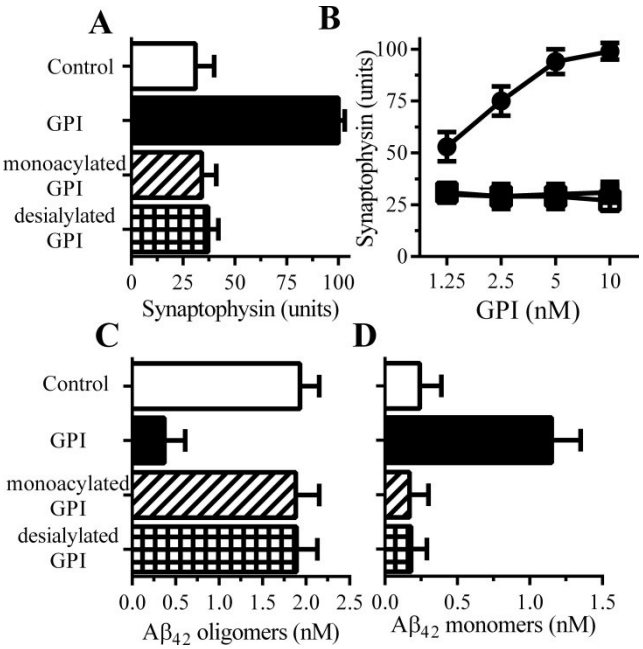

Figure 8. GPI-mediated suppression of toxic Aβ release is dependent upon sialic acid - (A) The amounts of synaptophysin in neurons incubated with CM from 7PA2 cells treated with control medium (□), 10 nM GPIs (■), 10 nM monoacylated GPIs (striped bars) or 10 nM desialylated GPIs (hatched bars). Values are means ± SD from triplicate experiments performed 3 times, *n*=9. (B) The amounts of synaptophysin in neurons incubated with CM from 7PA2 cells treated with GPIs (●), monoacylated GPIs (□) or desialylated GPIs (■) as shown. Values are means ± SD from triplicate experiments performed 3 times *n*=9. The concentrations of $A\beta_{42}$ oligomers (C) and $A\beta_{42}$ monomers (D) in CM from 7PA2 cells treated with control medium (□), 10 nM GPIs (■) 10 nM monoacylated GPIs (striped bars) or 10 nM desialylated GPIs (hatched bars). Values are means ± SD from triplicate experiments performed 3 times, *n*=9.

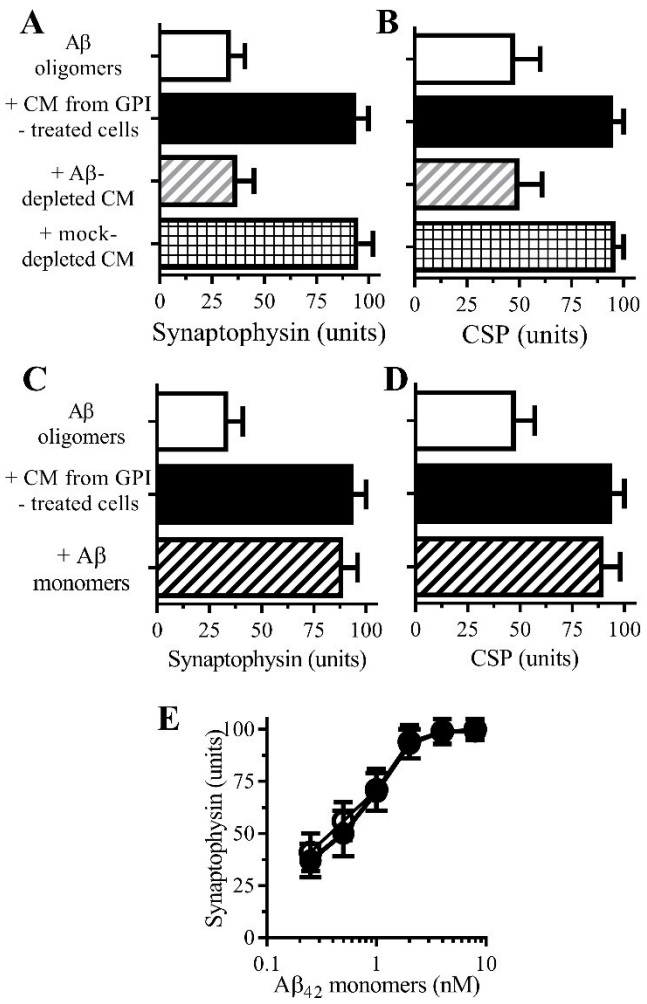

Figure 9. GPI-treated 7PA2 cells release neuroprotective Aβ monomers - The amounts of synaptophysin (A) and CSP (B) in neurons incubated with brain extract containing 2 nM $A\beta_{42}$ mixed with control medium (□), CM from 7PA2 cells treated with PrP^C-derived GPIs (■) or the same CM depleted of Aβ (striped bar) or mock-depleted (hatched bar). Values are means ± SD from triplicate experiments performed 3 times, *n*=9. The amounts of synaptophysin (C) and CSP (D) in neurons incubated with 1nM $A\beta_{42}$ mixed with control medium (□), CM from 7PA2 cells treated with PrP^C-derived GPIs (■), or monomers (striped bar) isolated from this CM. Values are means ± SD from triplicate experiments performed 3 times, *n*=9. (E) The amounts of synaptophysin in neurons pre-treated with Aβ monomers derived from 7PA2-CM (●) or from brain extract (○) as shown and incubated with brain extract containing 1 nM $A\beta_{42}$. Values are means ± SD from triplicate experiments performed 4 times, *n*=12.

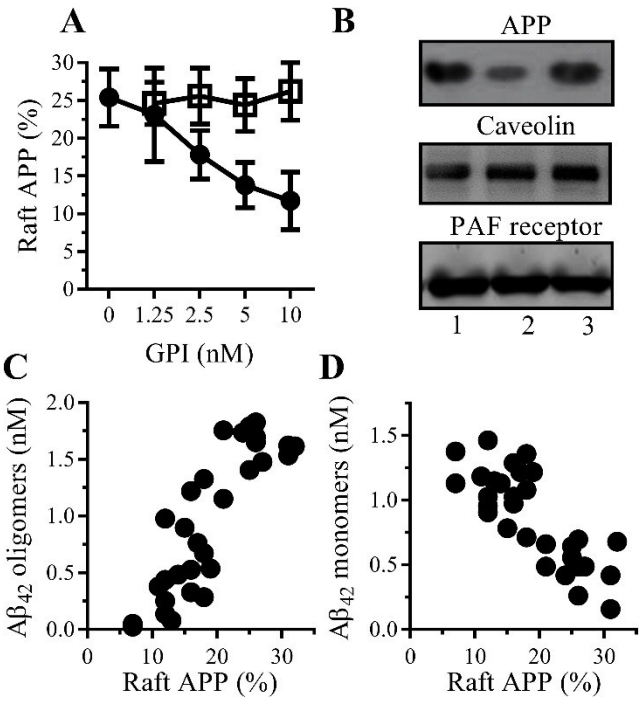

Figure 10 - GPIs reduced APP within lipid rafts – (A) The amounts of APP in DRMs (Rafts) in 7PA2 cells treated with GPIs (●) or desialylated GPIs (□) as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9. (B) Immunoblots showing the amounts of APP, caveolin and PAF receptors in DRMs (rafts) from 7PA2 cells treated with control medium (1), 10 nM GPIs (2) or 10 nM desialylated GPIs (3). (C) There was a significant correlation between the amounts of APP in lipid rafts (DRMs) and the concentrations of $A\beta_{42}$ oligomers found in CM from 7PA2 cells treated with GPIs (1.25 to 10 nM), Pearson's coefficient =0.84, $P<0.01$. (D). There was a significant inverse correlation between the amounts of APP in lipid rafts (DRMs) and the concentrations of $A\beta_{42}$ monomers found in CM from 7PA2 cells treated with GPIs (1.25 to 10 nM), Pearson's coefficient= -0.79, $P<0.01$.