Glimepiride protects neurons against amyloid-β-induced synapse damage

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Abstract

Alzheimer's disease is associated with the accumulation within the brain of amyloid-β (Aβ) peptides that damage synapses and affect memory acquisition. This process can be modelled by observing the effects of Aβ on synapses in cultured neurons. The addition of picomolar concentrations of soluble Aβ derived from brain extracts triggered the loss of synaptic proteins including synaptophysin, synapsin-1 and cysteine string protein from cultured neurons. Glimepiride, a sulphonylurea used for the treatment of diabetes, protected neurons against synapse damage induced by Aβ. The protective effects of glimepiride were multi-faceted. Glimepiride treatment was associated with altered synaptic membranes: a reduction in synaptic cholesterol following the loss of specific glycosylphosphatidylinositol (GPI)-anchored proteins including the cellular prion protein (PrP^C) that acts as a receptor for $A\beta_{42}$, increased synaptic gangliosides and altered cell signalling. More specifically, glimepiride reduced the Aβ-induced increase in cholesterol and the Aβ-induced activation of cytoplasmic phospholipase A_2 ($cPLA_2$) in synapses that occurred within cholesterol-dense membrane rafts. $A\beta_{42}$ binding to glimepiride-treated neurons was not targeted to membrane rafts and less $A\beta_{42}$ accumulated within synapses. These studies indicate that glimepiride modified the membrane micro-environments in which Aβ-induced signalling leads to synapse damage. In addition, soluble PrP^C, released from neurons by glimepiride, neutralised Aβ-induced synapse damage. Such observations raise the possibility that glimepiride may reduce synapse damage and hence delay the progression of cognitive decline in Alzheimer's disease.

Key words Alzheimer's disease, amyloid-β, glimepiride, glycosylphosphatidylinositols, phospholipase A_2, prion, synapses, synaptophysin.

1. Introduction

Alzheimer's disease (AD), a genetically heterogeneous disease that is the most common form of dementia, is a complex neurological disorder characterized by a progressive dementia (Selkoe, 2002; Tanzi, 2005). The amyloid hypothesis of AD pathogenesis maintains that the main event leading to AD is the production of toxic amyloid-β (Aβ) peptides derived from proteolytic cleavage of the amyloid precursor protein by β and γ secretases (De Strooper et al., 2010; Hardy and Selkoe, 2002). The cognitive decline in AD patients coincides with increasing concentrations of Aβ in the brain (Naslund et al., 2000) which leads to the subsequent disruption of neuronal processes, abnormal phosphorylation of tau and synapse damage. Some of the events that lead to neurodegeneration in AD can be examined by incubating cultured neurons with Aβ peptides.

In this study we measured concentrations of synaptic proteins in cultured primary cortical neurons incubated with Aβ preparations to study the molecular mechanisms involved in synapse degeneration. Since soluble Aβ oligomers that can diffuse throughout the brain are regarded as highly potent neurotoxins (Haass and Selkoe, 2007; Lambert et al., 1998) soluble forms of "natural Aβ" were isolated from brain extracts. The addition of soluble Aβ oligomers reduced the amounts of synaptophysin, synapsin-1, cysteine string protein (CSP) and vesicle-associated membrane protein (VAMP)-1 in cultured neurons indicative of synapse damage (Bate et al., 2010) thus providing a reliable tissue culture model of the synapse damage that is observed in AD. The biological effects of these Aβ oligomers occur at picomolar concentrations, similar to those in extracts from human brain or cerebrospinal fluid (Mc Donald et al., 2010; McLean et al., 1999; Wang et al., 1999).

Numerous biochemical, epidemiological, pharmacological and genetic studies demonstrated that cholesterol is a risk factor for the development of AD (Jick et al., 2000; Li et al., 2007; Puglielli et al., 2003). The requirement of cholesterol for the formation of specific membrane micro-domains called lipid rafts (Rajendran and Simons, 2005) is thought to be is a critical factor affecting AD pathogenesis. The observations that $A\beta_{42}$ accumulates within rafts (Kawarabayashi et al., 2004; Oshima et al., 2001) and that rafts are essential for the formation of signalling platforms (Mayor and Rao, 2004) suggests that $A\beta_{42}$ triggers the events that lead to neurotoxicity from within rafts. This hypothesis is supported by the observation that cholesterol depletion protected neurons against Aβ-induced neurodegeneration (Bate and Williams, 2007; Wang et al., 2001). However, cholesterol synthesis inhibitors are

Abbreviations

Alzheimer's disease (AD), amyloid-β (Aβ), cellular prion protein (PrP^C), chloromercuriphenylsulphonate (p-CMPS), cytoplasmic phospholipase A_2 ($cPLA_2$), cysteine-string protein (CSP), detergent-resistant memnbranes (DRM)s, di-methyl sulphoxide (DMSO), glycosylphosphatidylinositol (GPI), high performance thin-layer chromatography (HPTLC) , phosphatidylinositol (PI), phospholipase A_2-Activating Peptide (PLAP), phospholipase C (PLC), polyacrylamide gel electrophoresis (PAGE), prostaglandin (PG), vesicle-associated membrane protein (VAMP)-1.

regarded as crude pharmacological tools as cholesterol depletion also affects many other neuronal processes including the sensitivity of neurotransmitter receptors (Allen et al., 2007). The observation that rafts exist as multiple heterogeneous subsets containing different proteins and with different functions (Pike, 2004) raised the possibility that compounds that alter the function of specific rafts involved in Aβ-induced neurodegeneration could be discovered. The factors that affect the formation and function of rafts are inadequately understood. Rafts contain many proteins attached to cell membranes via glycosylphosphatidylinositol (GPI) anchors (Legler et al., 2005; Mayor and Riezman, 2004). As GPI anchors promote the formation of rafts (Brown and London, 2000), drugs that affect GPI anchors may consequently affect the composition and function of rafts.

Glimepiride, a sulphonylurea used to treat diabetes, activates an endogenous GPI-phospholipase C (GPI-PLC) (Movahedi and Hooper, 1997) leading to the release of GPI-anchored proteins including the cellular prion protein (PrP^C) that has been identified as a receptor that mediates Aβ-induced synapse damage (Lauren et al., 2009). Consequently glimepiride was reported to reduce membrane cholesterol (Bate et al., 2009) and affect the distribution of raft-resident proteins (Müller et al., 2005). Here we report that glimepiride protected neurons against Aβ-induced synapse damage. It did not affect the incorporation of $A\beta_{42}$ into neurons, rather it reduced the Aβ-induced changes in cell membranes and the activation of cytoplasmic phospholipase A_2 ($cPLA_2$) in synapses.

2. Materials and Methods

2.1. Primary neuronal cultures - Cortical neurons were prepared as described (Lesuisse and Martin, 2002). Neurons were plated (2×10^5 cells/well) in 48 well plates pre-coated with poly-L-lysine) in Ham's F12 containing 5% foetal calf serum for 2 hours. Cultures were shaken (600 r.p.m for 5 minutes) and non-adherent cells removed by 2 washes in PBS. Neurons were grown in neurobasal medium containing B27 components supplemented with nerve growth factor (5 nM) for 10 days. Immunohistochemistry showed that the cells were greater than 90% neurofilament positive. Neurons were subsequently pre-treated with test compounds including glimepiride, glipizide, p-chloromercuriphenylsulphonate (p-CMPS) or 0.2 units/ml phosphatidylinositol (PI)-PLC derived from *Bacillus cereus* (all from Sigma) for 1 hour before the addition of test samples including Aβ or Phospholipase A_2-Activating Peptide (PLAP) (Bachem) for 24 hours. All experiments were performed in accordance with European regulations (European Community Council Directive, 1986, 56/609/EEC).

2.2. Isolation of synaptosomes - Synaptosomes were prepared from cultured neurons on a discontinuous Percoll gradient as described (Thais et al., 2006; Westmark et al., 2011). Neurons were homogenized at 4°C in 1 ml of SED solution (0.32 M sucrose, 50 mM Tris-HCl, pH 7.2, 1 mM EDTA, and 1 mM dithiothreitol) and centrifuged at 1000 × *g* for 10 minutes. 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. Synaptosomes were collected from the interface of the 15% and 23% Percoll steps and washed in PBS before use. Isolated synaptosomes were pre-treated for 1 hour and incubated with peptides for 1 hour.

2.3. Cell/synaptosome extracts - Treated neurons/synaptosomes were washed 3 times with PBS and homogenised in a buffer containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.2% SDS, mixed protease inhibitors (4-(2-aminoethyl) benzenesulfonyl fluoride, Aprotinin, Leupeptin, Bestain, Pepstatin A and E-46) and a phosphatase inhibitor cocktail (PP1, PP2A, microcystin LR, cantharidin and p-bromotetramisole) (Sigma) at 10^6 cells/ml. Nuclei and cell debris were removed by centrifugation (300 x *g* for 5 minutes).

2.4. Isolation of detergent-resistant membranes (DRMs)/membrane rafts - Membrane rafts were isolated by their insolubility in non-ionic detergents. Briefly, samples 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 inhibitors and nuclei and large fragments were removed by centrifugation (300 x g for 5 minutes at 4ºC). The post nuclear supernatant was incubated on ice (4ºC) for 1 hour and centrifuged (16,000 x g for 30 minutes at 4ºC). The supernatant was reserved as the detergent soluble membrane (DSM) while the insoluble pellet was homogenised in an extraction buffer containing 10 mM Tris-HCL, pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.2% SDS and mixed protease inhibitors at 10^6 cells/ml, centrifuged (10 minutes at 16,000 x g) and the soluble material was reserved as the DRM fraction.

2.5. Western Blotting - Samples were mixed with Laemmli buffer, heated to 95ºC for 5 minutes and proteins were separated by electrophoresis on 15 or 12% polyacrylamide gels (PAGE). Proteins were transferred onto a Hybond-P PVDF membrane by semi-dry blotting. Membranes were blocked using 10% milk powder; PrP was detected by incubation with mAb ICSM18, synaptophysin with MAB368 (Abcam), CSP with rabbit polyclonal anti-CSP ((sc-33154) Santa Cruz), VAMP-1 with mAb 4H302 (Abcam), synapsin-1 with a rabbit polyclonal antibody (515200, Invitrogen), caveolin with rabbit polyclonal antibodies (Upstate) and Thy-1 with a rat mAb (Abcam). Bound antibodies were visualised using a combination of biotinylated anti-mouse/rat/rabbit IgG (Sigma), extravidin-peroxidase and enhanced chemiluminescence.

2.6. Isolation of PrP^C and Thy-1 – PrP^C and Thy-1 were prepared from neurons as described (Bate and Williams, 2012). Briefly, cells were homogenised in a buffer containing 10 mM Tris-HCl pH 7.4, 100 mM NaCl, 10 mM EDTA, 0.5 % Nonidet P-40, 0.5 % sodium deoxycholate and mixed protease inhibitors (as above). Cell debris was removed by centrifugation and soluble membrane preparations were passed over affinity columns loaded with mAbs to PrP^C (ICSM35) or anti-Thy-1 (Abcam). PrP^C and Thy-1 was eluted using glycine-HCl at pH 2.7, neutralised with 1 M Tris pH 7.4, desalted and concentrated. Soluble forms of PrP^C and Thy-1 were collected in the supernatants of neurons treated with 5 µM glimepiride. Supernatants were concentrated and desalted by centrifugation with a 3 kDa filter. Native and soluble proteins were isolated via reverse phase chromatography on C18 columns (Waters). For bioassays samples were solubilised in culture medium by sonication. The amounts of PrP^C in samples were measured by ELISA as described (Bate and Williams, 2011) and the amounts of Thy-1 by a bicinchoninic acid protein assay (Pierce). PrP^C preparations were analysed via high performance thin-layer chromatography (HPTLC) on silica gel 80 plates (Whatman). Samples were applied and developed using a mixture of choloroform/methanol/water (4/4/1 - v/v/v).

Plates were soaked in 0.1 % polyisobutyl methacrylate in hexane, dried, and blocked with 5 % milk powder. They were probed with 1 µg /ml of mAb ICSM18, washed with PBS-Tween, and incubated with goat anti–mouse IgG conjugated to peroxidase (Sigma) for 1 hour. The bound antibody was visualised using chemiluminescence.

2.7. Treatment of glimepiride-treated supernatants - Supernatants from glimepiride-treated neurons were incubated with 0.1 µg/ml mAb ICSM18 (reactive with PrP^{C}) and incubated at 4ºC on rollers for 1 hour. Protein G microbeads (Sigma) were added (10 µl/ml) for 30 minutes and protein G-antibody complexes were removed by centrifugation (16,000 x *g* for 10 minutes). Mock-depletions involved incubating supernatants with 0.1 µg/ml of an isotype control followed by protein G microbeads under the same conditions as above. Depleted media was filtered before further use.

2.8. Synaptophysin ELISA - The amounts of synaptophysin in neurons were measured by ELISA as described (Bate et al., 2010). Maxisorb immunoplates (Nunc) were coated with a mouse monoclonal antibody (mAb) to synaptophysin MAB368 (Millipore). Samples were applied 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. Absorbance was measured on a microplate reader at 405 nm. Samples were expressed as "units synaptophysin" where 100 units was defined as the amount of synaptophysin in 10^6 control neurons.

2.9. CSP ELISA – Maxisorb immunoplates were coated with a mAb to CSP ((sc-136468) Santa Cruz) and blocked with 5% milk powder. Samples were added 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 control cells.

2.10. Cholesterol - The concentrations of cholesterol in samples were measured using the Amplex Red cholesterol assay kit (Invitrogen), according to the manufacturer's instructions. Briefly, 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 550 nm and emission detection at 590 nm.

2.11. $cPLA_2$ ELISA - Maxisorb immunoplates were coated with 0.5 µg/ml of mAb anti-$cPLA_2$ (clone CH-7, Upstate) and blocked with 5% milk powder in PBS-tween. Samples were incubated for 1 hour and the amount of $cPLA_2$ was detected using a goat polyclonal anti-$cPLA_2$ (Santa-Cruz Biotech) followed by biotinylated anti-goat IgG, extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenyl phosphate solution. Absorbance was measured at 405 nm and the amount of $cPLA_2$ was calculated. The amount of $cPLA_2$ protein was expressed in units (100 units = amount of $cPLA_2$ in control preparations).

2.12. Activated $cPLA_2$ ELISA - The activation of $cPLA_2$ is accompanied by phosphorylation of the 505 serine residue which creates a unique epitope and can be measured by ELISA as described (Bate et al., 2010). Maxisorb immunoplates were coated with 0.5 µg/ml of mouse mAb anti-$cPLA_2$, clone CH-7 (Upstate) and blocked. Samples were incubated for 1 hour and the amount of activated $cPLA_2$ was detected using rabbit polyclonal anti-phospho-$cPLA_2$ (Cell Signalling Technology) followed by biotinylated anti-rabbit IgG (Sigma), extravidin-alkaline phosphatase and 1mg/ml 4-nitrophenyl phosphate solution. Results were expressed as "units activated $cPLA_2$" with 100 units defined as the amount of activated $cPLA_2$ in in control preparations.

2.13. Brain extracts – The temporal lobe from a 78 year old female with a clinical, and pathologically-confirmed, diagnosis of Alzheimer's disease, was supplied by Asterand, an international supplier of human tissue. Soluble extracts were prepared using methodology as previously described (Shankar et al., 2008). Briefly, brain tissue was cut into pieces of approximately 100 mg and added to 2 ml tubes containing lysing matrix D beads (Q-Bio). Neurobasal medium containing B27 components 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) to disrupt tissue. This process was performed 3 times and tubes were centrifuged at 16,000 x *g* for 10 minutes to remove cell debris. Soluble material was prepared by passage through a 30 kDa filter. The amounts of Aβ in each soluble extract were measured by ELISA (see below) and the supernatant aliquoted and stored at -80ºC. For immunoblot analysis, extracts were concentrated, mixed with an equal volume of 0.5% NP-40, 5 mM CHAPS, 50 mM Tris, pH 7.4 and separated by PAGE. Proteins were transferred onto a PVDF 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.

2.14. Immunodepletions - Brain extracts were incubated with 0.1 µg/ml mAb 4G8 (reactive with amino acids 17-24 of Aβ) and incubated at 4ºC on rollers for 1 hour. Protein G microbeads (Sigma) were added (10 µl/ml) for 30 minutes and protein G-antibody complexes were removed by centrifugation (16,000 x *g* for 10 minutes). Mock-depletions involved incubating brain extracts with 0.1 µg/ml of an isotype control followed by protein G microbeads under the same conditions as above. Depleted media was filtered before further use.

2.15. PrP^C ELISA - The amount of PrP^C in samples was determined by ELISA as described (Bate et al., 2010). Briefly, Maxisorb immunoplates were coated with mAb ICSM18 (D-Gen). Samples were added and bound PrP was detected with biotinylated mAb ICSM35 (D-Gen). Biotinylated mAb was detected using extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenyl phosphate solution. Absorbance was measured on a microplate reader at 405 nm and the amount of PrP in samples was calculated by reference to a standard curve of recombinant murine PrP (Prionics).

2.16. Aβ binding to PrP^C – Maxisorb immunoplates were coated with PrP^C or soluble PrP^C and blocked by the addition of 5% milk powder. Samples were added for 1 hour and bound Aβ was detected by the addition of 10 nM biotinylated mAb 6E10 (epitope = 1-16) (Covance) followed by 0.5 µg/ml of extravidin-alkaline phosphatase and 1

mg/ml 4-nitrophenyl phosphate solution. Absorbance was measured on a microplate reader at 405 nm and the amount of Aβ calculated by reference to a standard curve of synthetic $A\beta_{1-42}$ (Bachem).

2.17. Gangliosides – Synaptosomes were solubilised in chloroform/methanol/water (1/4/5). Extracts (5 µl) were applied to silica gel 60 high performance thin layer chromatography (HPTLC) plates (Whatman) and developed using a mixture of choloroform/methanol/water (4/4/1 - v/v/v). Plates were soaked in 0.1 % poly(isobutyl methacrylate) in hexane, dried, and blocked with PBS containing 5 % milk powder. Sialic acid containing lectins were detected by the addition of 1 µg/ml biotinylated *Sambucus nigra* lectin (Vector Labs) followed by extravidin peroxidase and detected with chemiluminescence. To quantify the amount of gangliosides in synaptosomes samples were diluted in propanol (1 in 100), 50 µl was plated into Nunc Maxisorb immunoplates and left to dry overnight. Plates were blocked with a 5% milk powder and gangliosides were detected by the addition of 1 µg/ml biotinylated *Sambucus nigra* lectin followed by extravidin alkaline phosphatase and 1 mg/ml 4-nitrophenyl phosphate solution. Absorbance was measured on a microplate reader at 405 nm.

2.18. Sample preparation - To detach Aβ42 from cellular binding proteins that could occlude specific epitopes cell extracts (200 µl) were mixed with 500 µl of 70% formic acid and sonicated. A 50 µl aliquot was added to 450 µl of 1 M Tris–HCl with protease inhibitors (as above) and sonicated before addition to ELISA.

2.19. $A\beta_{42}$ 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 (Sigma). Total Aβ was visualised by addition of 4-nitrophenyl phosphate solution. Absorbance was measured on a microplate reader at 405 nm and results were calculated by comparison to serial dilutions of synthetic $A\beta_{42}$.

2.20. Statistical Analysis - Comparison of treatment effects was carried out using Student's t-tests, Error bars are standard deviation (SD) and significance was set at $P<0.01$. Correlations were analysed using SPSS.

3. Results

3.1. Aβ triggers synapse damage in cultured neurons - The amounts of synaptic proteins in neurons incubated with Aβ was studied in a tissue culture model of AD-related synapse damage. An immunoblot showed that the brain extracts contained a mixture of Aβ monomers, dimers and trimers that were removed by depletion with the Aβ-reactive mAb G48 (Figure 1A). The addition of brain extracts to cultured neurons caused the loss of synaptic proteins including synapsin-1, VAMP-1, CSP and synaptophysin from cultured neurons (Figure 1B). However, brain extracts did not affect the amounts of caveolin, nor did it significantly reduce cell viability as measured by the thiazolyl blue tetrazolium (MTT) method (09% cell survival ± 6 compared with 100% ± 5, n=12, P=0.04). In order to quantify synapse damage ELISA specific for synaptophysin and CSP were used. Brain extracts caused a dose-dependent

rduction in the amounts of both synaptophysin and CSP within neurons (Figures 1C & D). While brain extracts contain Aβ it was not clear whether it was Aβ that was responsible for the synapse damage in these neuronal cultures. Immunodepletion with mAb G48 reduced concentrations of both $A\beta_{40}$ (4.9 nM ± 0.3 to 0.3 nM ± 0.3 nM) and $A\beta_{42}$ (1.2 nM ± 0.16 to 0.11 nM ± 0.1 nM) in brain extracts, whereas mock-depletion had no significant effect on either $A\beta_{40}$ (4.9 nM ± 0.3 to 4.7 nM ± 0.3 nM) and $A\beta_{42}$ (1.2 nM ± 0.16 to 1.1 nM ± 0.1 nM). The addition of brain extract that had been depleted of Aβ did not trigger the loss of synaptophysin of CSP from neurons indicating that the toxic entity was Aβ and consistent with the hypothesis that Aβ is a major neurotoxin in AD brains. Notably, the synapse damage was triggered by brain extract containing nanomolar/picomolar concentrations of $A\beta_{42}$, concentrations similar to those observed in CSF (Mc Donald et al., 2010; McLean et al., 1999; Wang et al., 1999). As $A\beta_{42}$ is considered to be more neurotoxic than $A\beta_{40}$ in all further studies the amount of brain extract added was standardized on the amount of $A\beta_{42}$ it contained.

3.2. Glimepiride reduced Aβ-induced synapse damage – Since the identification of compounds that protect neurons against Aβ-induced synapse damage is a rational strategy to treat AD the effects of glimepiride on neurons were studied. Glipizide, another sulphonylurea used to treat diabetes, was used as a control. Firstly we found that the addition of 5 μM glimepiride or glipizide alone did not alter the amounts of synaptophysin or CSP in neurons indicating that these drugs did not cause synapse damage. Next, pre-treatment with 5 μM glimepiride, but not glipizide, protected neurons against the Aβ-induced loss of synaptophysin or CSP (Figures 2A and B). The protective effect of glimepiride was dose-dependent (Figure 2C). To determine whether the continued presence of glimepiride was essential for its protective effects, neurons were treated with 5 μM glimepiride for 1 hour and then washed 3 times prior to the addition of Aβ for a further 24 hours. Neurons treated in this way remained partially resistant to Aβ (Figure 2D). Glimepiride-treated neurons were not protected against all toxins; the loss of synaptophysin and CSP from neurons incubated with PLAP was not affected by pre-treatment with 5 μM glimepiride (Figures 2E & F).

3.3. Digestion with PI-PLC protected neurons against Aβ-induced synapse damage – Some of the effects of glimepiride are mediated by activation an endogenous GPI-PLC (Müller et al., 1994). As this is an effect of glimepiride that was not shared by glipizide we hypothesised that activation of GPI-PLC was the key factor responsible for the protective effect of glimepiride. Two methods were used to test this hypothesis. Firstly, neurons treated with PI-PLC showed less synapse damage when incubated with Aβ than did control neurons (Figures 4A & B). Next, we sought to reverse the effect of glimepiride by the use of p-CMPS, an inhibitor of GPI-PLC (Stanton et al., 2002). The addition of 200 μM p-CMPS alone did not affect the synaptophysin content of neurons (97 units ± 8 compared to 100 ± 7, n=9, P=0.7) or affect synapse damage induced by Aβ. However, the addition of 200 μM p-CMPS reversed the protective effect of 5 μM glimepiride. Thus, the synaptophysin content of neurons incubated with Aβ was higher when pre-treated with 5 μM glimepiride than when pre-treated with a combination of 5 μM glimepiride and 200 μM p-CMPS (Figures 3C & D). These results are consistent with the hypothesis that the activation of GPI-PLC was responsible for the neuroprotective effect of glimepiride.

Since the activation of GPI-PLC can release some GPI-anchored proteins from cells, the possibility that glimepiride affected cellular receptors for Aβ and hence the binding of $A\beta_{42}$ to neurons was studied. Neurons treated with 5 μM glimepiride or digested with PI-PLC were incubated with 5 nM $A\beta_{42}$ for 1 hour. There were no significant differences in the concentrations of $A\beta_{42}$ found in neurons between control and glimepiride-treated neurons (4.68 nM $A\beta_{42}$ ± 0.28 compared with 4.64 nM ± 0.27, n=9, P=0.81) or between control and PI-PLC digested neurons (4.68 nM $A\beta_{42}$ ± 0.282 compared with 4.52 nM ± 0.25, n=9, P=0.26).

3.4. Glimepiride reduced the expression of PrP^C at synapses – The effects of glimepiride upon synapses were studied by isolating synaptosomes from neurons treated with glimepiride for 1 hour. The protein, synaptophysin and CSP content of synaptosomes from control and glimepiride-treated neurons were not significantly different. The cellular prion protein (PrP^C), identified as a receptor for $A\beta_{42}$ (Lauren et al., 2009), is attached to membranes via a GPI anchor (Stahl et al., 1987) and released by the addition of glimepiride (Bate et al., 2009). Treatment of neurons with glimepiride, but not glipizide, caused a dose-dependent reduction in the concentrations of PrP^C in synaptosomes (Figure 4A). Treatment with glimepiride was selective; it did not affect the amounts of the GPI-anchored Thy-1, nor did affect the amounts of caveolin or VAMP-1 in synaptosomes (Figure 4B). The glimepiride-induced reduction in of PrP^C in synaptosomes was reduced by the inclusion of 200 μM p-CMPS which increased the concentration of synaptic PrP^C from 0.3 ± 0.2 nM to 1.78 ± 0.27 nM, n=9, P<0.05. When treated synaptosomes were analysed by HPTLC, glimepiride-treated synaptosomes were found to contain more gangliosides than control synaptosomes (Figure 4C). Glimepiride caused a dose-dependent increase in the ganglioside content of synapses; the amounts of gangliosides in synaptosomes showed a significant inverse correlation with concentrations of PrP^C, Pearson's coefficient=-0.801, P<0.01 (Figure 4D).

Next, the effects of glimepiride on the binding of Aβ to synapses were tested using isolated synaptosomes. Pre-treatment with 5 μM glimepiride did not significantly alter the amounts of $A\beta_{42}$ that bound to synaptosomes; there were no significant difference in the concentrations of $A\beta_{42}$ in control and treated synaptosomes after incubation with 1 nM $A\beta_{42}$ (0.9 nM $A\beta_{42}$ ± 0.07 compared with 0.89 nM ± 0.09, P=0.59, n=9). Such results indicate that PrP^C is not the only receptor that can bind $A\beta_{42}$ expressed at synapses. Since $A\beta_{42}$ is found within cholesterol-dense rafts in brain and neuronal extracts (Kawarabayashi et al., 2004; Lee et al., 1998), the effect of glimepiride on the binding of $A\beta_{42}$ to rafts was examined. When synaptosomes were incubated for 1 hour with 1 nM $A\beta_{42}$, most of the $A\beta_{42}$ was found in rafts (DRMs). Pre-treatment of synaptosomes with 5 μM glimepiride resulted in a significant reduction in the concentrations of $A\beta_{42}$ found in DRMs and a corresponding increase in DSMs (Figure 4E). This effect of glimepiride was blocked by inhibiting endogenous GPI-PLCs with the inclusion of p-CMPS.

3.5. Aβ binds to soluble PrP^C – Treatment of neurons with glimepiride causes the release of soluble PrP^C into culture supernatants (Bate et al., 2009). The soluble PrP^C in these supernatants was collected using an immunoaffinity column followed by low pressure reverse phase chromatography using C18 columns. PrP^C eluted from C18 columns in concentrations of propanol between 60 and 75% whereas the soluble PrP^C eluted at lower concentrations of propanol (Figure 5A). PrP^C and soluble PrP^C had different migration patterns in HPTLC consistent with a significant change in

hydrophobicity (Figure 5B). Since changes in the GPI anchor can alter the structure of some proteins (Barboni et al., 1995) we tested whether Aβ bound to soluble PrP^C. PrP^C and soluble PrP^C were immobilised on ELISA plates and incubated with brain extract. Aβ bound to both PrP^C and soluble PrP^C, but not to soluble Thy-1, a GPI-anchored protein used as a control (Figure 5C). Aβ did not bind to soluble PrP^C preparations from which PrP^C had been immunodepleted (Figure 5D) indicating that Aβ was binding to soluble PrP^C and not a contaminant.

3.6. Soluble PrP^C blocked Aβ-induced synapse damage - The concept that disease-relevant conformations of Aβ may constitute only a small proportion of total Aβ (Glabe, 2008) raises the possibility that soluble PrP^C might bind to the non-toxic forms of Aβ rather than toxic Aβ. This hypothesis was tested by examining the effects of soluble PrP^C upon Aβ-induced synapse damage. First, Aβ was mixed with supernatants from glimepiride-treated neurons that contained soluble PrP^C. The presence of this supernatant reduced the Aβ-induced synapse damage (Figure 6A & B). Supernatants from which soluble PrP^C had been removed by immunodepletion did not block Aβ-induced synapse damage indicating that the protective component of the supernatant was soluble PrP^C. To confirm the role of soluble PrP^C as an inhibitor of Aβ-induced synapse damage soluble PrP^C was isolated on C18 columns. The Aβ-induced synapse damage was reduced by the addition of 10 nM soluble PrP^C but was not affected by 10 nM soluble Thy-1 (Figure 6C). To ensure that the effects of soluble PrP^C were not mediated by a direct effect upon neurons, neurons were pre-treated with 10 nM soluble PrP^C for 1 hour. They were then washed 3 times and incubated with Aβ. Under these conditions the soluble PrP^C did not affect Aβ-induced synapse damage (Figure 6D) thus confirming that toxic conformations of Aβ bound to soluble PrP^C. To determine efficacy, serial dilutions of soluble PrP^C were incubated with 500 pM $Aβ_{42}$. Soluble PrP^C, but not soluble Thy-1, protected neurons against Aβ-induced synapse damage in a dose-dependent manner (Figure 6E). The neuroprotective effect of soluble PrP^C was stimulus specific as the presence of 10 nM soluble PrP^C did not affect PLAP-induced synapse damage (Figure 6F).

3.7. Glimepiride reduced Aβ-induced activation of $cPLA_2$ in synapses - Several studies suggest that aberrant activation of $cPLA_2$ is involved in Aβ-induced synapse damage. For example, Aβ peptides activate PLA_2 (Shelat et al., 2008; Zhu et al., 2006) and inhibition of $cPLA_2$ prevented Aβ-induced synapse damage *in vitro* (Bate et al., 2010) and *in vivo* (Desbene et al., 2012). Here we show that the addition of brain extract increased the amounts of activated $cPLA_2$ in synapses (Figure 7A). Immunodepletion studies showed that Aβ was responsible for the activation of synaptic $cPLA_2$. Pre-treatment of synaptosomes with 5 μM glimepiride, but not glipizide, reduced the Aβ-induced activation of $cPLA_2$ (Figure 7B). The effects of glimepiride upon the activation of $cPLA_2$ in synaptosomes were stimulus specific as it did not affect the activation of $cPLA_2$ by PLAP (Figure 7C) indicating that glimepiride did not directly inhibit $cPLA_2$. The presence of soluble PrP^C did not activate synaptic $cPLA_2$ but pre-treatment with 10 nM soluble PrP^C significantly reduced the Aβ-induced activation of $cPLA_2$ (Figure 7D).

Upon activation, $cPLA_2$ translocate to specific intracellular membranes utilizing an N-terminal, calcium-dependent lipid binding domain (Nalefski et al., 1994). Isolation of DRMs (lipid rafts) was used to quantify the change in synaptic location of $cPLA_2$ in response to Aβ. In control synaptosomes less than 20% of $cPLA_2$ was found within DRMs. The

addition of 1 nM $A\beta_{42}$ significantly increased the amounts of $cPLA_2$ found in DRMs in control synaptosomes. Pre-treatment of synaptosomes with glimepiride significantly reduced the Aβ-induced translocation of $cPLA_2$ into DRMs (Figure 7E). The activation of $cPLA_2$ is the first step in the production of prostaglandins (PG) including PGE_2 which causes synapse damage in cultured neurons (Bate et al., 2010). Here we show that pre-treatment of synaptosomes with 5 μM glimepiride reduced Aβ-induced PGE_2 production, but had no effect upon PLAP-induced PGE_2 production (Figure 7F).

3.8. Glimepiride reduced the Aβ-induced increase in cholesterol in synapses - Our finding that the addition of brain extracts significantly increased the concentrations of cholesterol within synaptosomes (Figure 8A) is consistent with reports that increased cholesterol was found in Aβ positive synapses in the cortex of AD brains (Gylys et al., 2007). The concentrations of cholesterol in synaptosomes were not affected by the addition of Aβ-depleted brain extract indicating that Aβ was the molecule causing the increase in cholesterol. There was a significant correlation between cholesterol concentrations and activated $cPLA_2$ in synaptosomes incubated with $A\beta_{42}$ (1 to .125 nM), Pearson's coefficient=0.734, $P<0.01$ (Figure 8B). Pre-treatment with glimepiride or PI-PLC blocked the Aβ-induced increase in cholesterol in synaptosomes (Figure 8C). The addition of 200 μM p-CMPS reversed the effect of 5 μM glimepiride upon the Aβ-induced increase in synaptic cholesterol (Figure 8D). Pre-treatment of Aβ with supernatants from glimepiride-treated neurons reduced the Aβ-induced increase in cholesterol, an effect that was dependent upon soluble PrP^C (Figure 8E). Similarly, pre-treatment of Aβ with 10 nM soluble PrP^C, but not 10 nM soluble Thy-1, blocked the Aβ-induced increase in cholesterol in synapses (Figure 8F).

4. Discussion

The identification of drugs that protect neurons against Aβ-induced synapse damage is a rational strategy to reduce the cognitive decline that is observed in AD patients. There is increasing interest in drugs used in the clinic to treat other diseases, whose side effects and pharmacokinetics are well known, to be prescribed "off-label" as AD treatments, especially those that cross the blood-brain barrier. Here we show that glimepiride affected some of the molecular mechanisms by which Aβ causes neurodegeneration in tissue culture models of AD pathogenesis. The key finding, that physiologically relevant concentrations glimepiride (Becker et al., 2007) protected neurons against Aβ-induced synapse damage, was associated with subtle membrane changes at synapses including the loss of PrP^C and reduced Aβ-induced increases in cholesterol and activated $cPLA_2$.

Low nanomolar/picomolar concentrations of Aβ triggered the loss of synaptic proteins including synaptophysin and CSP from cultured cortical neurons indicating synapse degeneration. These concentrations of Aβ are similar to those found the brains of AD patients (Mc Donald et al., 2010; McLean et al., 1999; Wang et al., 1999). Glimepiride did not completely block Aβ-induced synapse damage; rather it increased the concentrations of Aβ required to cause synapse

damage by approximately 10 fold. Neurons treated with glimepiride were not protected against synapse damage-induced by PLAP indicating that it affected a stimulus-selective pathway.

Sulphonylureas such as glimepiride mimic the effects of insulin upon cells. Like many drugs glimepiride has a broad range of actions and while most of the anti-diabetic effects of glimepiride are shared by glipizide, neuroprotection was a glimepiride-specific effect. Since glimepiride, but not glipizide, activates an endogenous GPI-PLC (Müller et al., 2001) we hypothesised that this was the molecular basis of the protective effects of glimepiride. This hypothesis was supported by two observations; firstly that neurons treated with PI-PLC were also protected against Aβ-induced synapse damage and secondly that the protective effect of glimepiride was reversed by a selective inhibitor of GPI-PLC.

Although glimepiride reduced the expression of PrP^C (Bate et al., 2009), which acts as a receptor for $A\beta_{42}$ (Lauren et al., 2009), similar amounts of $A\beta_{42}$ bound to control and glimepiride-treated neurons or synaptosomes. $A\beta_{42}$ has been reported to bind to multiple receptors including the amyloid precursor protein (Lorenzo et al., 2000) and α7 nicotinic acetylcholine and glutamate receptors (Renner et al., 2010; Wang et al., 2000) and it seems likely that $A\beta_{42}$ binds to some of these in glimepiride-treated neurons/synaptosomes. We noted that glimepiride significantly reduced the amounts of $A\beta_{42}$ targeted to rafts and subsequently the amounts of $A\beta_{42}$ that accumulated within synapses. As $A\beta_{42}$ accumulated at synapses in Prnp knockout neurons (Bate and Williams, 2011), but not in glimepiride-treated neurons, we concluded that glimepiride did more than simply removing PrP^C from neurons. The observation that glimepiride did not affect the binding of $A\beta_{42}$ to isolated synaptosomes suggested that in neuronal cultures $A\beta_{42}$ binds to receptors on the cell perikyra and subsequently traffics to synapses, a process that is inhibited by glimepiride.

Both lipid rafts and Aβ-induced neurodegeneration are sensitive to cholesterol depletion suggesting that the molecular processes leading to neurodegeneration are generated from within cholesterol-sensitive rafts (Bate and Williams, 2007; Wang et al., 2001; Williamson et al., 2008). Since cholesterol has an important role in the synapse, the inhibition of cholesterol synthesis is not a feasible approach to disrupt Aβ-induced neurodegeneration. Aβ affects cholesterol homeostasis (Grimm et al., 2007) and here we showed that Aβ increased the concentrations of cholesterol in synaptosomes; an observation that is consistent with reports of increased cholesterol found in Aβ-positive synapses in the cortex of AD brains (Gylys et al., 2007). Such observations are compatible with the hypothesis that in normal synapses Aβ creates the lipid rafts responsible for synapse degeneration. The oligomerisation of GPI-anchored proteins triggers raft formation and the increase in synaptic cholesterol was coincident with Aβ mediated cross-linkage of raft-associated PrP^C (Taylor and Hooper, 2006). Our observation that glimepiride did not affect the amount of $A\beta_{42}$ that bound to synaptosomes, but blocked the Aβ-induced increase in synaptic cholesterol indicates that the presence

of $A\beta_{42}$ *per se* does not increase cholesterol. We hypothesise that the binding of Aβ to receptors other than PrP^C in glimepiride-treated synaptosomes does not trigger an increase in membrane cholesterol.

Lipid rafts are thought to exist as multiple, heterogeneous sub-sets that have different protein cargos and have different functions (Pike, 2004). Although glimepiride reduced the amounts of PrP^C in synaptosomes, it did not affect rafts containing Thy-1, caveolin or VAMP-1 and consequently had a more selective effect than cholesterol synthesis inhibitors. Thus, glimepiride appeared to selectively affect the formation of those rafts that mediate Aβ-induced synapse damage. Lipid rafts are dynamic in nature and adapt readily to changes in membrane composition (Pike, 2004). The inverse correlation between the amounts of PrP^C and gangliosides in synapses suggested that PrP^C may suppress ganglioside expression. Thus the biochemical changes induced in synapses by glimepiride may be protective due to the removal of PrP^C, forcing Aβ to bind to alternative receptors, or by increasing gangliosides which suppress toxicity.

Precisely how Aβ triggers synapse damage is unclear. PrP^C is associated with several signaling molecules including tyrosine kinases (Mouillet-Richard et al., 2000) and $cPLA_2$ (Bate and Williams, 2011). The aberrant activation of $cPLA_2$ is thought to underlie the pathology of AD based on reports that $A\beta_{42}$ activates $cPLA_2$ (Shelat et al., 2008; Zhu et al., 2006) and that inhibition of $cPLA_2$ reduced the Aβ-induced synapse damage *in vitro* (Bate et al., 2010) and *in vivo* (Desbene et al., 2012). In addition, $cPLA_2$ inhibitors ameliorate the cognitive decline seen in a transgenic model of AD (Sanchez-Mejia et al., 2008). Critically the Aβ-induced activation of $cPLA_2$ in synapses was cholesterol-sensitive (Bate and Williams, 2007). An emerging paradigm is that of cell activation occurring as a consequence of multiple individual rafts coalescing to form a membrane platform capable of sustained cell activation (Pike, 2004). PrP^C acts as a scaffold protein that organises signalling platforms (Linden et al., 2012) and activated $cPLA_2$ was found within PrP^C-containing rafts (Bate and Williams, 2011). The dynamic nature of raft composition (Pike, 2004) is consistent with the hypothesis that critical concentrations of $A\beta_{42}$ triggered the formation of a raft platform capable of the sustained activation of $cPLA_2$ that leads to synapse damage. In glimepiride-treated synapses most of the $cPLA_2$ remained in the cytoplasm and the Aβ-induced activation of $cPLA_2$ was reduced. Initially it was thought that the glimepiride-induced increase in gangliosides inhibited $cPLA_2$ (Nakamura et al., 2010) but that this hypothesis is unlikely as glimepiride did not inhibit PLAP-induced activation of synaptic $cPLA_2$. We therefore conclude that $A\beta_{42}$ binding to glimepiride-treated synaptosomes does so via receptors that do not trigger a membrane platform which captures and activates $cPLA_2$.

Soluble PrP^C was released from glimepiride-treated neurons (Bate et al., 2009) and bound Aβ oligomers. Critically, supernatants from glimepiride-treated neurons blocked Aβ-induced synapse damage; an activity that was dependent upon PrP^C. Soluble PrP^C also reduced both the Aβ-induced increases in synaptic cholesterol and activation of $cPLA_2$. The protective effect of soluble PrP^C was stimulus specific and did not affect synapse damage induced by PLAP. These results suggest that should soluble PrP^C be generated in the brain it may be able to neutralise the toxic Aβ oligomers that are responsible for synapse failure, and hence the dementia associated with AD. In this regard it is of

interest that in a transgenic mouse model of AD containing APPPS1^{+} Prnp$^{0/0}$ and crossed with mice producing anchorless PrP^{C} (Chesebro et al., 2005) the APPPS1-related suppression of LTP was inhibited; an effect that was independent of any effects upon the production of $A\beta_{42}$ (Calella et al., 2010). Many GPI-anchored proteins are found in soluble forms and although soluble PrP^{C} is released by platelets (Perini et al., 1996) we are not aware of studies demonstrating soluble PrP^{C} within the brain. Although we did not detect soluble PrP^{C} in mouse brain extracts (unpublished data) it may be possible to generate enough soluble PrP^{C} within the brain by treatment with glimepiride to neutralise the soluble Aβ oligomers responsible for synapse damage.

5. Conclusions

We report that glimepiride protected neurons against Aβ-induced synapse damage. The protective effect of glimepiride was associated with multiple factors including the loss of PrP^{C} from synapses, the production of soluble PrP^{C}, and the altered distribution of cholesterol, $A\beta_{42}$ and $cPLA_2$ within cell membranes. In glimepiride-treated neurons, $A\beta_{42}$ was not targeted to lipid rafts and did not trigger synapse damage. Furthermore, in glimepiride-treated synaptosomes Aβ did not increase cholesterol concentrations and failed to activate $cPLA_2$; observations indicating that glimepiride targets rafts involved in Aβ-induced synapse damage. As glimepiride is already used in the clinic and can cross the blood-brain barrier, it could be considered as a novel adjunctive treatment that could reduce the pathogenesis of AD.

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6. Figures

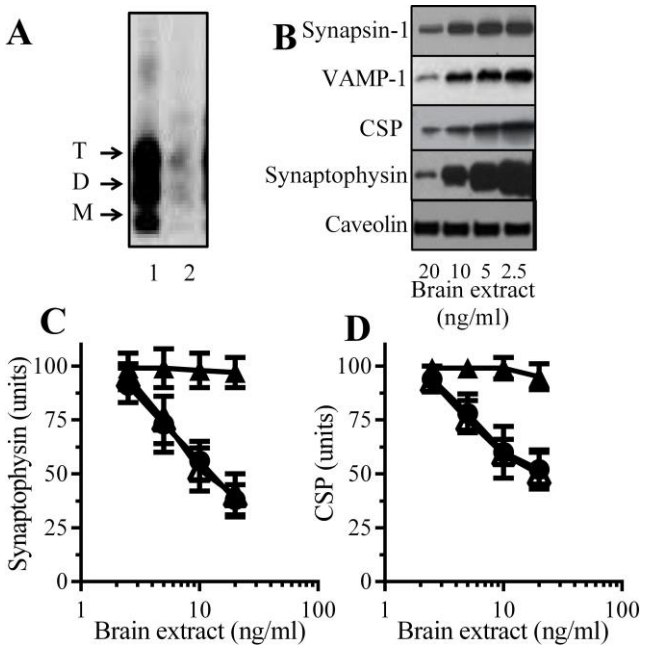

Figure 1. Aβ damages synapses in cultured neurons - (A) Immunoblot showing Aβ monomers (M), dimers (D) and trimers (T) in brain extract (1) and Aβ-depleted brain extrtact (2). (B) Immunoblots showing the amounts of synapsin-1, VAMP-1, CSP, synaptophysin and caveolin in neurons incubated with brain extract as shown. The amounts of synaptophysin (D) and CSP (E) in neurons incubated with brain extract (●), Aβ-depleted brain extract () or mock-depleted brain extract (○). Values are means ± SD from triplicate experiments performed 3 times (n=9).

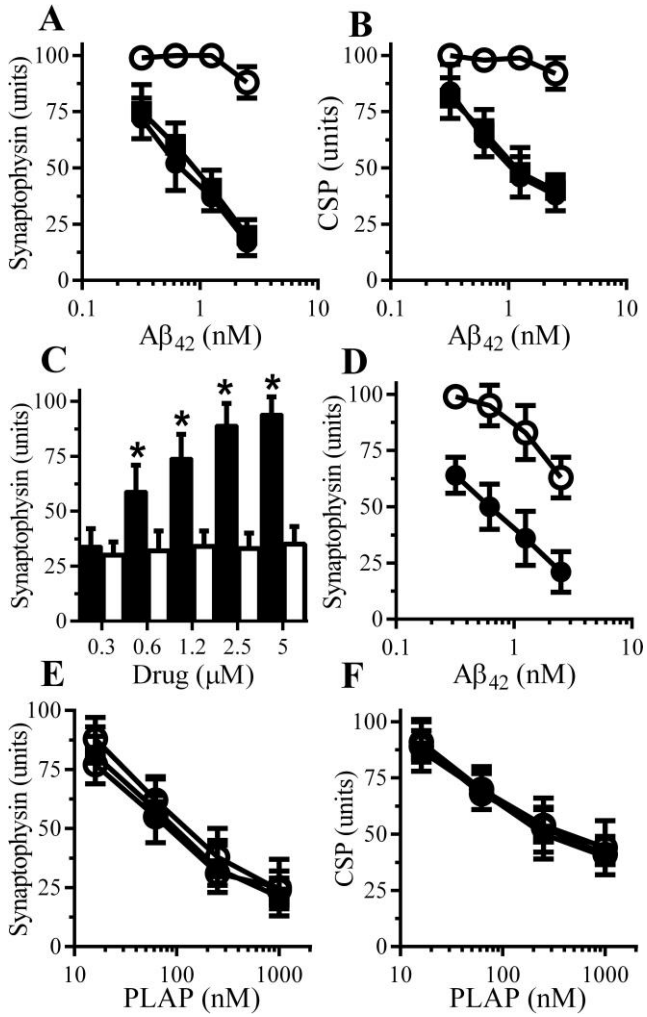

Figure 2. Glimepiride protected neurons against Aβ-induced synapse damage - The amounts of synaptophysin (A) and CSP (B) in neurons pre-treated with control medium (●), 5 μM glimepiride (○) or 5 μM glipizide (■) and incubated with $A\beta_{42}$ as shown. Values are means ± SD from triplicate experiments performed 3 times (n=9). Values are means ± SD from triplicate experiments performed 3 times (n=9). (C) The amounts of synaptophysin in neurons pre-treated with glimepiride (■) or glipizide (□) as shown and incubated with 2 nM $A\beta_{42}$. Values are means ± SD from triplicate experiments performed 3 times, n=9. *=synaptophysin significantly higher than control neurons incubated with Aβ, $P<0.05$. (D) The amounts of synaptophysin in neurons pre-treated with a control medium (●) or 5 μM glimepiride (○) and washed. Washed neurons were then incubated with $A\beta_{42}$ as shown. Values are means ± SD from triplicate experiments performed 4 times (n=12). The amounts of synaptophysin (E) and CSP (F) in neurons pre-treated with control medium (●), 5 μM glimepiride (○) or 5 μM glipizide (■) and incubated with PLAP. Values are means ± SD from triplicate experiments performed 3 times (n=9).

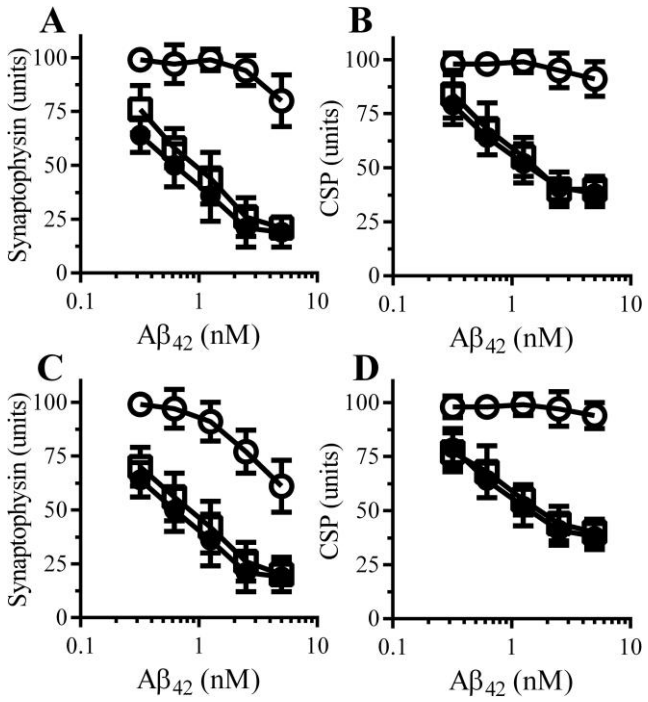

Figure 3. Digestion with PI-PLC protected neurons against Aβ-induced synapse damage - (A) The amounts of synaptophysin (A) and CSP (B) in neurons pre-treated with control medium (●), 0.2 units/ml PI-PLC (○) or heat-denatured PI-PLC (□) and incubated with $A\beta_{42}$ as shown. Values are means ± SD from triplicate experiments performed 3 times (n=9). The amounts of synaptophysin (C) and CSP (D) in neurons pre-treated with a control medium (●), 5 μM glimepiride (○), 200 μM p-CMPS (■) or a combination of 5 μM glimepiride and 200 μM pCMPS (□) and incubated with $A\beta_{42}$ as shown. Values are means ± SD from triplicate experiments performed 3 times (n=9).

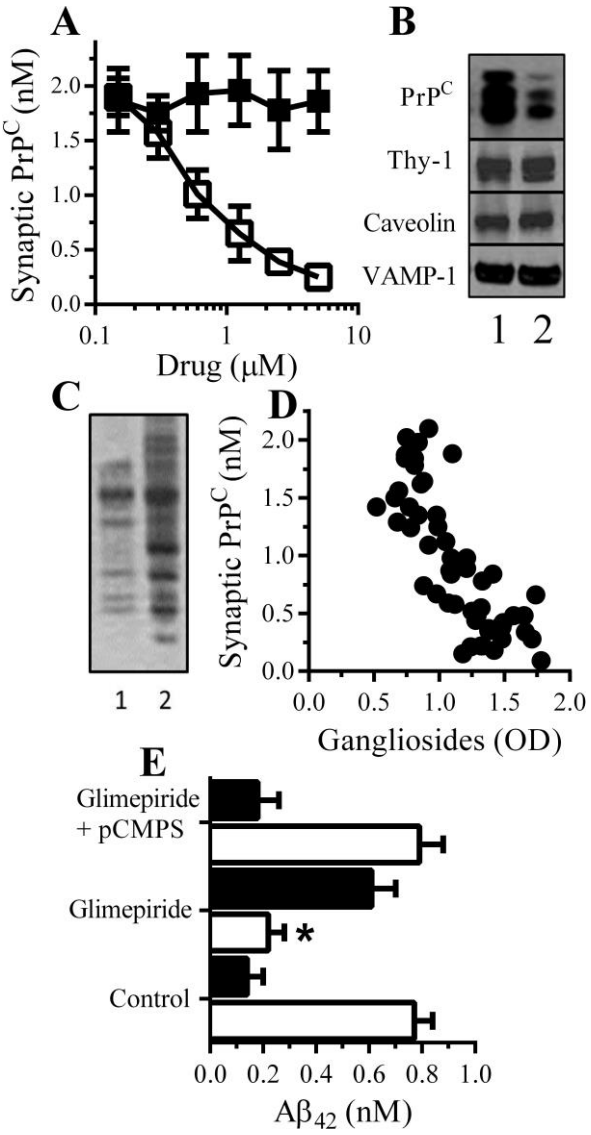

Figure 4. Glimepiride altered the composition of synapses – (A) The concentrations of PrP^C in synaptosomes derived from neurons treated for 1 hour with glimepiride (□) or glipizide (■) as shown. Values are means ± SD from triplicate experiments performed 3 times, n=9. (B) Immunoblots showing the amounts of PrP^C, Thy-1, caveolin and VAMP-1 in synaptosomes incubated with control medium (1) or 5 μM glimepiride (2) for 1 hour. (C) Neurons were treated with 5 μM glimepiride for 1 hour and synaptosomes prepared. Butanol extracts were separated on HPTLC plates and sialylated gangliosides detected using biotinylated *S. nigra.* (D) There was a significant inverse correlation between amounts of PrP^C and sialylated gangliosides in synaptosomes prepared from neurons treated for 1 hour with glimepiride (5 μM to 0.625 μM), Pearson's coefficient = -0.801, P<0.01. (E) Synaptosomes pre-treated with control medium, 5 μM glimepiride or 5 μM glimepiride and 200 μM pCMPS were incubated with 1 nM Aβ$_{42}$ for 1 hour and the amounts of Aβ$_{42}$ in DRMs (rafts) (□) and DSM (■) measured. Values are means ± SD from triplicate experiments performed 3 times, n=9. *=Aβ$_{42}$ significantly lower than in DRMs from control synaptosomes, P<0.05.

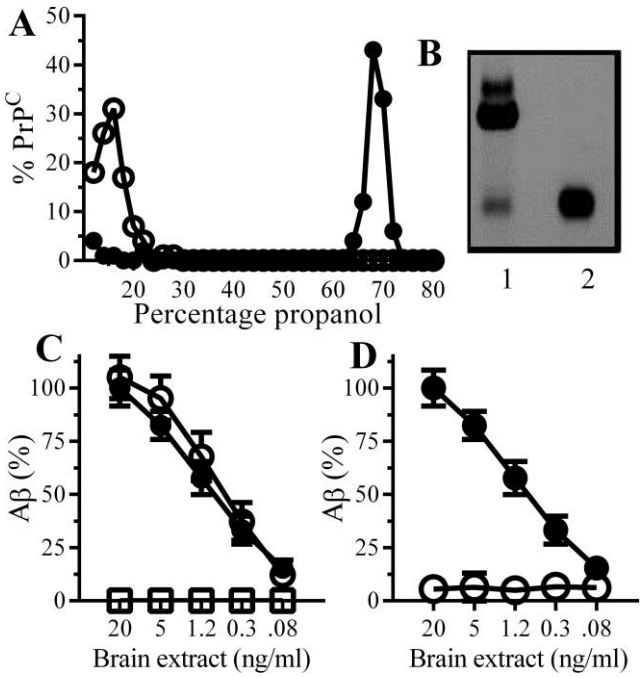

Figure 5 – Glimepiride generates soluble PrP^C that binds Aβ – (A) The amounts/concentrations of PrP^C (●) or soluble PrP^C (○) in fractions eluted from C18 columns under a gradient of propanol and water. Values are means of duplicates. (B) PrP^C (1) and soluble PrP^C (2) separated on silica gel 60 HPTLC plates. (C) The amounts of Aβ bound to immunoplates coated with 50 nM PrP^C (●), 50 nM soluble PrP^C (○) or 50 nM soluble Thy-1 (□) and incubated with brain extract as shown. Values are means (% maximum OD) ± SD from triplicate experiments performed 4 times (n=12). (D) The amounts of Aβ bound to immunoplates coated with a preparation containing 50 nM soluble PrP^C (●) or the same soluble PrP^C preparation that had been immunodepleted of PrP^C (○) and incubated with brain extract as shown. Values are mean Aβ (% maximum OD) ± SD from triplicate experiments performed 4 times (n=12).

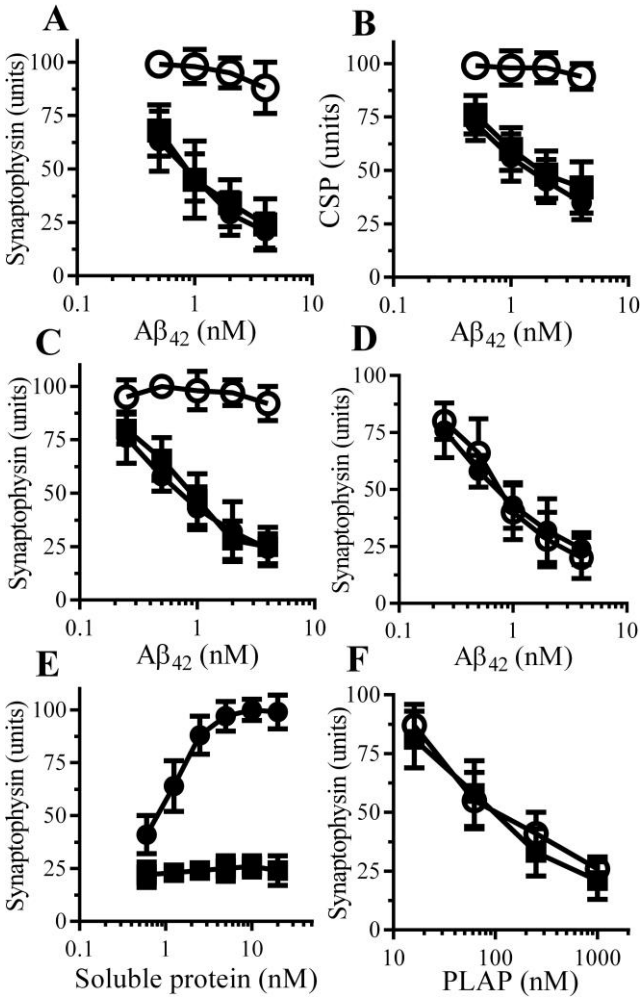

Figure 6 - Soluble PrP^C protected neurons against Aβ-induced synapse damage – The amounts of synaptophysin (A) and CSP (B) in neurons incubated with Aβ pre-treated with control medium (●), supernatants from glimepiride-treated neurons (○) or the same supernatants that had been PrP^C-depleted (■). Values are means ± SD from triplicate experiments performed 4 times, n=12. (C) The amounts of synaptophysin in neurons incubated Aβ pre-treated with control medium (●), 10 nM soluble PrP^C (○) or 10 nM soluble Thy-1 (■). Values are means ± SD from triplicate experiments performed 4 times, n=12. (D) The amounts of synaptophysin in neurons pre-treated with control medium (●) or 10 nM soluble PrP^C (○), washed 3 times and incubated with $A\beta_{42}$ as shown. Values are means ± SD from triplicate experiments performed 4 times, n=12. (E) The amounts of synaptophysin in neurons incubated with 4 nM $A\beta_{42}$ that had been pre-treated with soluble PrP^C (●) or soluble Thy-1 (■) as shown. Values are means ± SD from triplicate experiments performed 4 times, n=12. (F) The amounts of synaptophysin in neurons incubated with PLAP pre-treated with control medium (●) or 10 nM soluble PrP^C (○). Values are means ± SD, from triplicate experiments performed 3 times, n=9.

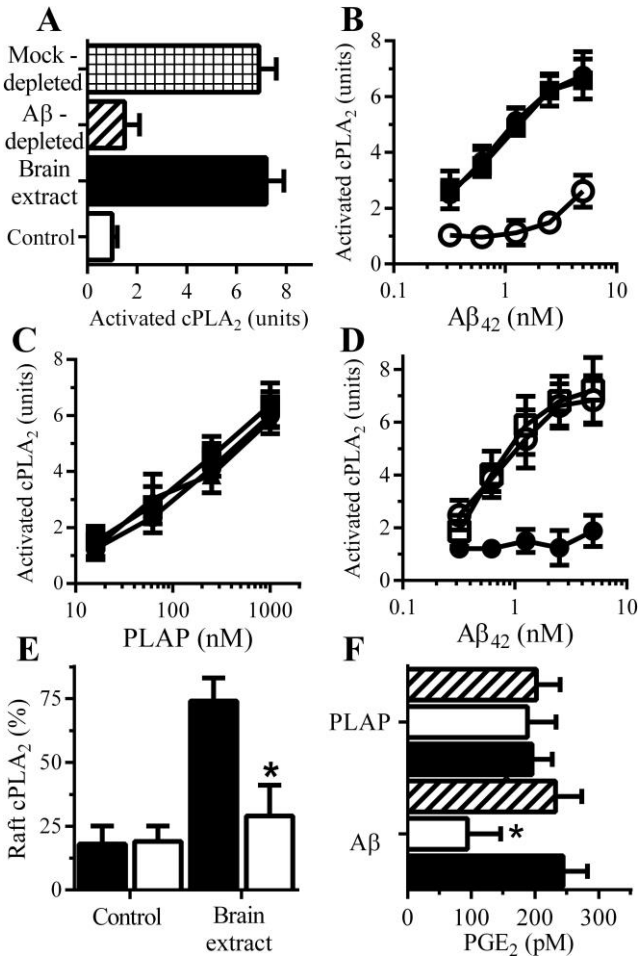

Figure 7. Glimepiride reduced the activation of cPLA$_2$ by Aβ – (A) The amounts of activated cPLA$_2$ in synaptosomes incubated with control medium (□), brain extract (■), Aβ-depleted brain extract (striped bar) or mock-depleted brain extract (hatched bar) as shown. Values are means ± SD from triplicate experiments repeated 3 times, n=9. (B) The amounts of activated cPLA$_2$ in synaptosomes pre-treated control medium (●), 5 μM glimepiride (○) or 5 μM glipizide (■) and incubated with Aβ$_{42}$ as shown. Values are means ± SD from triplicate experiments repeated 3 times, n=9. (C) The amounts of activated cPLA$_2$ in synaptosomes pre-treated with control medium (●), 5 μM glimepiride (○) or 5 μM glipizide (■) and incubated with PLAP as shown. Values are means ± SD from triplicate experiments repeated 3 times, n=9. (D) The amounts of activated cPLA$_2$ in synaptosomes incubated with Aβ$_{42}$ pre-treated with control medium (○), 10 nM soluble PrP^C (●) or 10 nM soluble Thy-1 (□). Values are means ± SD from triplicate experiments performed 4 times (n=12). (E) The amounts of cPLA$_2$ within lipid rafts (DRMs) from synaptosomes pre-treated with control medium (■) or 5 μM glimepiride (□) and incubated with 1 nM Aβ$_{42}$. Values are means ± SD from triplicate experiments repeated 3 times, n=9. *=significantly less cPLA$_2$ in lipid rafts than in control synaptosomes incubated with Aβ, $P<0.05$. (F) The concentrations of PGE$_2$ produced by synaptosomes pre-treated with control medium (■), 5 μM glimepiride (□) or 5 μM glipizide (striped bar) and incubated with Aβ or PLAP. Values are means ± SD from triplicate experiments performed 2 times, (n=6). *=PGE$_2$ significantly less than control synaptosomes incubated with peptide.

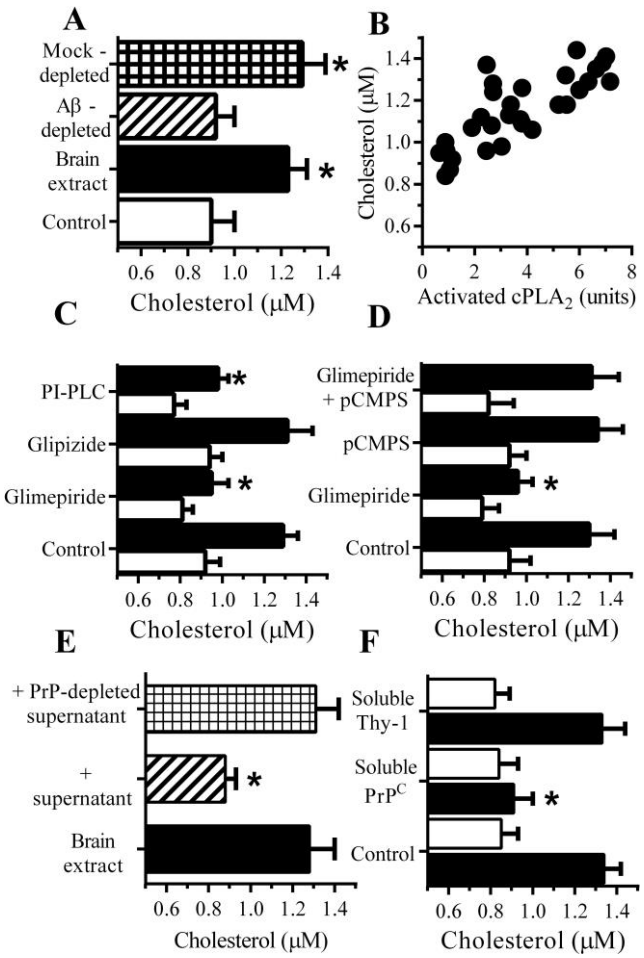

Figure 8. Glimepiride reduces the Aβ induced increase in synaptic cholesterol - (A) The concentrations of cholesterol in synaptosomes incubated with control medium (□), brain extract (■), Aβ-depleted brain extract (striped bar) or mock-depleted brain extract (hatched bar). Values are means ± SD from quadruplicate experiments performed twice (n=8). *=cholesterol significantly higher than in control synaptosomes, $P<0.05$. (B) There was a significant correlation between the concentrations of cholesterol and amounts of activated $cPLA_2$ in synaptosomes incubated for 1 hour with $A\beta_{42}$ (1 to .125 nM), Pearson's coefficient=0.734, $P<0.01$. (C) The concentrations of cholesterol in synaptosomes pre-treated with control medium, 5 μM glimepiride, 5 μM glipizide or 0.2 units/ml PI-PLC and incubated with control medium (□) or 1 nM $A\beta_{42}$ (■). Values are means ± SD from triplicate experiments performed 3 times (n=9). *=cholesterol significantly lower than synaptosomes treated with Aβ, $P<0.05$. (D) The concentrations of cholesterol in synaptosomes pre-treated with control medium, 5 μM glimepiride, 200 μM pCMPS or glimepiride and pCMPS and incubated with control medium (□) or 1 nM $A\beta_{42}$ (■). Values are means ± SD from triplicate experiments performed 3 times (n=9). *=cholesterol significantly lower than control synaptosomes incubated with Aβ, $P<0.05$. (E) The concentrations of cholesterol in synaptosomes incubated with brain extract (■), brain extract mixed with the supernatant from glimepiride-treated neurons (striped bar) or brain extract mixed with the same supernatant immunodepleted of PrP^C (hatched bar). Values are means ± SD from triplicate experiments performed 3 times (n=9). *=cholesterol significantly lower than control synaptosomes treated with brain extract, $P<0.05$. (F) The concentrations of cholesterol in synaptosomes incubated with brain extract

(■) or control medium (□) mixed with control medium, 10 nM soluble PrP^C or 10 nM soluble Thy-1 (striped bars). Values are means ± SD from triplicate experiments performed 4 times (n=12). *=cholesterol significantly lower than control synaptosomes treated with brain extract, $P<0.05$.

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