An *in vitro* model for synaptic loss in neurodegenerative diseases suggests a neuroprotective role for valproic acid via inhibition of $cPLA_2$ dependent signalling

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Running title – Valproic acid protects against synapse damage

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Abbreviations

Alzheimer's disease (AD), amyloid-β (Aβ), α-synuclein (αSN), cellular prion protein (PrP^C), cholesterol ester hydrolase (CEH), cytoplasmic phospholipase A_2 ($cPLA_2$), cysteine-string protein (CSP), decanoic acid (DA), di-methyl sulphoxide (DMSO), phospholipase A_2-Activating Peptide (PLAP), polyacrylamide gel electrophoresis (PAGE), propylisopropylacetic acid (PIA), prostaglandin (PG), valproic acid (VPA), vesicle-associated membrane protein (VAMP)-1.

Abstract

Many neurodegenerative diseases present the loss of synapses as a common pathological feature. Here we have employed an *in vitro* model for synaptic loss to investigate the molecular mechanism of a therapeutic treatment, valproic acid (VPA). We show that amyloid-β (Aβ), isolated from patient tissue and thought to be the causative agent of Alzheimer's disease, caused the loss of synaptic proteins including synaptophysin, synapsin-1 and cysteine-string protein from cultured mouse neurons. Aβ-induced synapse damage was reduced by pre-treatment with physiologically relevant concentrations of VPA (10 μM) and a structural variant propylisopropylacetic acid (PIA). These drugs also reduced synaptic damage induced by other neurodegenerative-associated proteins α-synuclein, linked to Lewy body dementia and Parkinson's disease, and the prion-derived peptide PrP82-146. Consistent with these effects, synaptic vesicle recycling was also inhibited by these proteins and protected by VPA and PIA. We show a mechanism for this damage through aberrant activation of cytoplasmic phospholipase A_2 ($cPLA_2$) that is reduced by both drugs. Furthermore, Aβ-dependent $cPLA_2$ activation correlates with its accumulation in lipid rafts, and is likely to be caused by elevated cholesterol (stabilising rafts) and decreased cholesterol ester levels, and this mechanism is reduced by VPA and PIA. Such observations suggest that VPA and PIA may provide protection against synaptic damage that occurs during Alzheimer's and Parkinson's and prion diseases.

Introduction

Neurodegenerative diseases comprise a disparate group of conditions involving a common loss of synaptic function. These conditions provide a huge societal impact, with a large number of people experiencing severe symptoms. Understanding the molecular processes involved in these diseases and the development of either existing treatments (with new indications) or new treatment thus provides an urgent need. Alzheimer's disease is amongst the most important of these diseases, where the progressive loss of neuronal function is associated with the production of neurotoxic amyloid-β (Aβ) peptides that are cleaved from the C terminal of the amyloid precursor protein (Hardy, 2006). The clinical symptoms in Alzheimer's disease are caused by the loss or dysfunction of synapses (Walsh and Selkoe, 2004), and the best correlate of the degree of dementia in Alzheimer's disease patients is a reduction in synaptic density and the loss of synaptic proteins including synaptophysin, cysteine-string protein (CSP), vesicle-associated membrane protein (VAMP)-1 and synapsin-1 (Reddy et al., 2005; Terry et al., 1991). Yet the mechanism leading to synaptic loss remains unclear.

Other neurodegenerative disorders are also associated with synaptic loss, including Lewy body dementia, Parkinson's disease (Kramer and Schulz-Schaeffer, 2007) and prion diseases (Ferrer, 2002). *In vitro* models for these diseases employ aggregated α-synuclein protein (αSN) that accumulates at synaptic terminals, or the prion-derived peptide PrP82-146 (Salmona et al., 2003) to cause synaptic damage in cultured neurons (Bate et al., 2010). Such neurotoxic peptides have been shown to cause a reduction in synaptic density as determined by quantifying the amounts of synaptophysin and CSP. The molecular and cellular mechanisms involved in αSN and PrP82-146-dependent synaptic degeneration also remains poorly understood.

To bypass the long and expensive process of developing new therapeutic agents for neurological disorders (Wegener and Rujescu 2013), it may be possible to re-purpose compounds with established safety profiles. One such compound, valproic acid (2-propylpentanoic acid (VPA)), is a short branched-chain fatty acid

that has been proposed to have potential therapeutic role in Alzheimer's disease treatment (Qing et al., 2008a) and neuroprotection (Zhang et al., 2014). VPA is primarily used for the treatment of epilepsy and bipolar disorder treatment, although it has numerous molecular mechanisms and effects (Terbach and Williams, 2009), including the inhibition of $cPLA_2$ (Bazinet et al., 2006) and the regulation of lipid homeostasis (Elphick et al., 2012). Here we show that synapse damage caused by Aβ, αSN and PrP82-146 is reduced by VPA and a structural derivative (propylisopropylacetic acid (PIA)). We show the mechanism for this neuroprotective effect occurs through the inhibition of Aβ, αSN and PrP82-146 dependent cytoplasmic phospholipase A_2 (PLA_2) hyperactivation. We further demonstrate that this mechanism of Aβ-induced toxicity is associated with increased translocation of $cPLA_2$ to lipid rafts (leading to hyperactivation), since Aβ causes a dose-dependent increase in cholesterol and a reduction in cholesterol esters, and that this process is reduced by VPA and PIA.

Materials and Methods

Brain extracts – Soluble brain extracts from an Alzheimer's patient containing Aβ of similar in size and potency to the Aβ species previously isolated from the brains of Alzheimer's disease patients (Shankar et al., 2008) were used. Extracts were derived from the temporal lobe of a 78 year old female with a clinical, and pathologically-confirmed, diagnosis of Alzheimer's disease, supplied by Asterand, an international supplier of human tissue. 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). This process was performed 3 times before tubes were centrifuged at 16,000 x *g* for 10 minutes to remove cell debris. Soluble material was prepared by passage through a 50 kDa filter (Sartorius) (16,000 x *g* for 30 minutes) to remove any protease activity. The amounts of Aβ in each soluble extract were measured by ELISA (see below) and the supernatant aliquoted and stored at -80°C. For immunoblot analysis, brain extracts were mixed with an equal volume of 0.5% NP-40, 5 mM CHAPS, 50

mM Tris, pH 7.4 and separated by polyacrylamide gel electrophoresis (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. The amounts of $A\beta_{42}$ in preparations were determined by ELISA.

Size exclusion chromatography (SEC) – Soluble brain extracts were concentrated using desalting columns (3 kDa filter – Sartorius). These preparations were injected into a Superdex 75 PC column (separates peptides ranging from 3 kDa to 70 kDa) (GE Healthcare) and eluted at a rate of 0.2 ml/minute. Fractions were collected and tested by Aβ ELISA (see below).

Immunodepletions - Brain extracts were incubated with 0.1 μg/ml mAb 4G8 (reactive with amino acids 17-24 of Aβ) or isotype controls (mock depletions) and incubated at 4ºC on rollers for 1 hour. Protein G microbeads were added (10 μl/ml) (Sigma) for 30 minutes and protein G bound-antibody complexes removed by centrifugation.

Primary neuronal cultures - Primary cortical neurons were prepared from the brains of mouse embryos (day 15.5) after mechanical dissociation. Neurons were plated at 5×10^5 cells/well in 48 well plates in Hams F12 containing 5% foetal calf serum for 2 hrs. Cultures were shaken (600 r.p.m for 5 mins) and non-adherent cells removed by 2 washes in phosphate buffered saline (PBS). Neurons were subsequently grown in neurobasal medium containing B27 components (PAA) for 10 days. Immunostaining showed that after 10 days culture less than 5% of the viable cells stained for glial fibrillary acidic protein or F4/80 (astrocytes or microglial cells). Neurons were subsequently pre-treated with test compounds including VPA, PIA and decanoic acid for 1 hour before the addition of test samples including Aβ, recombinant human α-synuclein (αSN) (Sigma), PrP82-146 (Salmona et al., 2003)(a gift from Professor M Salmona, Milan) 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) and approved by the local authority veterinary service/ethical committee.

Cell extracts - Treated cells were washed twice in PBS and homogenised in 10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate and 0.2% SDS at 10^6 cells/ml. Mixed protease inhibitors (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, Aprotinin, Leupeptin, Bestatin, Pepstatin A and E-46) (Sigma) and a phosphatase inhibitor cocktail including PP1, PP2A, microcystin LR, cantharidin and p-bromotetramisole (Sigma) were added and nuclei and large fragments were removed by centrifugation (1000 x *g* for 5 minutes).

Western Blotting - Samples were mixed with Laemmli buffer containing β-mercaptoethanol, heated to 95ºC for 5 minutes and proteins were separated by electrophoresis on 15% polyacrylamide gels. Proteins were transferred onto a Hybond-P polyvinylidene difluoride 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), VAMP-1 with mAb 4H302 (Abcam), caveolin with rabbit polyclonal antibodies (Upstate) and CSP with rabbit polyclonal anti-CSP ((sc-33154) Santa Cruz). These were visualised using combinations of biotinylated anti-mouse/goat/rat/rabbit IgG (Sigma), extravidin-peroxidase and enhanced chemiluminescence.

Synaptophysin ELISA. The amount of synaptophysin in neuronal extracts was measured by ELISA as described (Bate et al., 2010). Maxisorb immunoplates (Nunc) were coated with a mouse anti-synaptophysin mAb (MAB368 - Chemicon) as a capture antibody and bound synaptophysin was detected using rabbit polyclonal anti-synaptophysin (Abcam) followed by a biotinylated anti-rabbit IgG (Abcam), extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenol phosphate (pNPP) solution (Sigma). 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 untreated cells.

CSP ELISA – Maxisorb immunoplates were coated with a monoclonal antibody (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.

Isolation of synaptosomes - Synaptosomes were prepared on a discontinuous Percoll gradient based on methods previously described (Thais et al., 2006). Briefly, 10^6 neurons were homogenized at 4 °C in 1 ml of SED solution (0.32 M sucrose, 50 mM Tris-HCl pH 7.4, 1 mM EDTA and 1 mM dithiothreitol) and centrifuged at 1000 × *g* for 5 minutes. The supernatant was transferred to a 4-step gradient of 3, 10, 15 and 23% Percoll in SED solution and centrifuged at 16,000 × *g* for 30 minutes at 4ºC. The synaptosomes were collected from the interface of the 15% and 23% Percoll steps and washed twice at 4ºC. Freshly prepared synaptosomes were pre-treated with drugs for 1 hour and incubated with peptides for 1 hour.

Synaptic vesicle recycling - The uptake of the fluorescent dye FM1-43 (Molecular probes) into synaptic recycling vesicles was used to determine synaptic activity. Treated synaptosomes were pulsed with 5 μM FM1-43 and 1 μM ionomycin (Sigma) for 5 minutes, washed 3 times in ice cold PBS and homogenised in methanol. Extracts were transferred into 96 well black microplates (Sterilin) and fluorescence was measured in a spectrophotometer using excitation at 480 nm and measuring emission at 625 nm. Samples were expressed as "% fluorescence" where 100% fluorescence was defined as the amount of fluorescence in control synaptosomes.

Isolation of lipid rafts (detergent-resistant membranes) - These membranes were isolated by their insolubility in non-ionic detergents. Briefly, synaptosomes 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 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.

Sucrose density gradients - Synaptosomes were homogenised in a buffer containing 250 mM sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM EGTA, mixed protease inhibitors and 1 mM dithiothreitol. Particulate membrane fragments and nuclei were removed by centrifugation (1000 x *g* for 5 min). Membranes were washed by centrifugation at 16,000 x *g* for 10 minutes at 4ºC and suspended in an ice-cold buffer containing 1% Triton X-100, 10 mM Tris-HCl pH 7.2, 150 mM NaCl, 10 mM EDTA. 5–40% sucrose solutions were prepared and layered to produce a gradient. Solubilised membranes were layered on top and centrifuged at 50000 x *g* for 18 hours at 4°C. Serial 1 ml aliquots were collected from the bottom of gradients.

$cPLA_2$ ELISA/activated $cPLA_2$/PGE_2 measurement - The amounts of $cPLA_2$ in extracts was measured by ELISA as described (Bate and Williams, 2011). Maxisorb immunoplates were coated with 0.5 µg/ml of mouse mAb anti-$cPLA_2$ (clone CH-7 - Upstate) and blocked with 5% milk powder in PBS + 0.1% tween 20 (PBST). Samples were incubated for 1 hour and the amount of bound $cPLA_2$ 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 pNPP solution. Absorbance was measured at 405 nm and the amount of $cPLA_2$ protein expressed in units, 100 units = amount of $cPLA_2$ in control preparations. 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 (Bate et al., 2010). To measure the amount of activated $cPLA_2$, an ELISA using a mAb (anti-$cPLA_2$, clone CH-7) combined with rabbit polyclonal anti-phospho-$cPLA_2$ (Cell Signalling Technology) followed by biotinylated anti-rabbit IgG (Sigma), extravidin-alkaline phosphatase and 1mg/ml pNPP solution. Absorbance was measured on a microplate reader at 405 nm. Results were expressed as "units activated $cPLA_2$" (100 units = amount of activated $cPLA_2$ in control preparations). The amounts of PGE_2 in synaptosomes were determined using a competitive enzyme immunoassay kit (Amersham Biotech, Amersham, UK) according to the manufacturer's instructions.

$A\beta_{42}$ 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). Bound $A\beta_{42}$ was visualised by addition of 4-nitrophenol phosphate solution. Absorbance was measured at 405 nm and compared to titrations of synthetic $A\beta_{1\text{-}42}$ (Bachem).

Cholesterol content - The amounts 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. By performing the assay in the presence or absence of cholesterol esterase (50 units/ml) (Sigma) the assay can also determine the amounts of esterified cholesterol within samples.

Drugs – VPA and decanoic acid was obtained from Sigma. PIA was obtained from Ukrogsyntez Ltd. Stock solutions were dissolved in ethanol or di-methyl sulphoxide (DMSO) and diluted in medium to obtain final working concentrations. Vehicle controls consisted of equal dilutions of ethanol or DMSO.

Statistical Methods - Differences between treatment groups were assessed using Student's paired t tests. Error bars are standard deviation (SD). Correlations were bivariate analysis and significance was set at 0.01%

Results

Brain extracts contain toxic Aβ - Synaptic degeneration is a prominent feature of the early stages of Alzheimer's disease (Reddy et al., 2005) and is thought to be caused by a toxic soup of Aβ peptides including $A\beta_{40}$, $A\beta_{42}$ and $A\beta_{43}$ (Benilova et al., 2012). To develop an *in vitro* model for synaptic loss, we employed brain extracts derived from a patient with Alzheimer's disease containing a mixture of Aβ monomers, dimers and trimers, as characterised by Western blot (Figure 1A) and by size exclusion chromatography (Figure 1B). We then examined the effect of increasing concentrations of Aβ over a 24 hour period on synapses from cultured primary mouse cortical neurons. Addition of increasing amounts of Aβ-containing brain extracts triggered the loss of synaptophysin (Figure 1C) and CSP (Figure 1D) and other synaptic proteins including VAMP-1 and synapsin-1 indicative of synapse damage (Figure 1E). These effects occurred in the absence of any neuronal death as measured by thiazolyl blue tetrazolium (101% cell survival ± 6 compared with 100% ± 5, n=6, P=0.6) and there was no loss of caveolin from treated neurons. Immunodepletion of brain extract with mAb G48 reduced concentrations of both $A\beta_{40}$ (4.9 nM ± 0.3 compared with 0.3 nM ± 0.3 nM, n=9, P<0.01) and $A\beta_{42}$ (1.42 nM ± 0.16 to 0.11 nM ± 0.1 nM, n=9, P<0.01). Immunodepleted brain extract did not cause any significant loss of synaptophysin or CSP from neurons indicating that Aβ was the synaptotoxic entity in these preparations. Mock-depletions, with control mouse IgG, did not affect the concentration of either $A\beta_{40}$ (4.9 nM ± 0.3 compared with 4.74 nM ± 0.9, n=9, P=0.58) or $A\beta_{42}$ (1.42 nM ± 0.16 compared with 1.38 ± 0.37 nM, n=9, P=0.48), nor did they affect the brain extract-induced reduction of neuronal synaptophysin or CSP.

Aβ, αSN and prion-induced synapse damage was reduced by VPA and PIA – To investigate a role of VPA on cultured neurons, synaptophysin and CSP levels were measured in neurons in the absence or presence of increasing amounts of Aβ-containing brain extracts for a 24 hour period. Pre-treatment of neurons with VPA (10 μM, 1 hour) did not significantly alter the amounts of synaptophysin (Figure 2A) or CSP (Figure 2B) in neurons, indicating that VPA did not affect synapses in the absence of pathogenic signalling. However, pre-treatment of neurons with VPA (10 μM) for 1 hour prior to incubation with increasing concentrations of Aβ-containing brain extracts for a 24 hour period, gave rise to a dose-

dependent neuroprotective effect in blocking the loss of synaptophysin (Figure 2A) or CSP (Figure 2B). This effect was also shown with a structurally related VPA analogue, propylisopropylacetic acid (PIA), but not with a straight-chain fatty acid, decanoic acid (DA). Neither PIA, nor decanoic acid affected synaptophysin or CSP concentrations in the absence of Aβ (supplementary data, Table 1). The effects of VPA and PIA were dose-dependent (Figure 2C).

Other neurodegenerative diseases including Lewy body dementia, Parkinson's disease and prion diseases are also associated with synaptic loss (Kramer and Schulz-Schaeffer, 2007). In these diseases, aggregated αSN and the disease-associated, prion-derived peptide (PrP82-146) cause synapse damage. To examine a role for VPA and PIA in protection against these neurodegenerative-associated proteins, cultured neurons were initially exposed to αSN and the prion-derived peptide PrP82-146 for a 24 hour period, and synaptic protein loss was monitored. We show increasing concentrations of αSN also triggered the loss of synaptophysin (Figure 3A) and CSP (Figure 3B) in cultured neurons. However, pre-treatment with either VPA or PIA, but not decanoic acid (all at 10 μM) for 1 hour prior to αSN exposure, partially protected against the loss of synaptophysin (Figure 3A) or CSP (Figure 3B). Similarly, increasing concentrations of PrP82-146 for a 24 hour period also triggered the loss of synaptophysin (Figure 3A) and CSP (Figure 3B), and pre-treatment with VPA or PIA, but not 10 μM decanoic acid (both at 10 μM) for 1 hour prior to PrP82-146 exposure also partially protected against the loss of synaptophysin (Figure 3C) or CSP (Figure 3D). In contrast, pre-treatment with VPA, PIA or decanoic acid (all at 10 μM) did not alter the loss of synaptophysin or CSP-induced by phospholipase A_2-activating peptide (PLAP) (Figure 3E &F) suggesting a specificity of effect relating to neurodegenerative protein function.

Aβ, αSN and prion-induced reduction in synaptic vesicle recycling was reduced by VPA and PIA - The fluorescent dye FM1-43 is used to visualise synaptic vesicles uptake in confocal microscopy (Klingauf et al., 1998). This system was modified to enable quantification of FM1-43 uptake into synaptosomes (multiple synapses). We showed that ionomycin (a calcium ionophore that stimulates neurotransmitter release and synaptic vesicle recycling) caused the uptake of FM1-43 by synaptosomes (Figure 4A)

consistent with increased vesicle recycling. Using this approach, vesicle recycling was not affected by pre-treating synaptosomes with VPA, PIA or decanoic acid (all at 10 μM) in the absence of Aβ. However, the addition of Aβ-containing brain extract to synaptosomes reduced recycling in a dose-dependent manner (Figure 4B) and Aβ-depleted brain extract did not affect recycling, indicating that Aβ was responsible for this effect. The inhibitory effect of Aβ was reduced in synaptosomes pre-treated with VPA or PIA for 1 hour, but was not affected by decanoic acid (all at 10 μM; Figure 4C). Similarly, pre-treatment of synaptosomes with VPA or PIA, but not decanoic acid significantly reduced the inhibitory effects of PrP82-146 (Figure 4D) and αSN (Figure 4E) on the vesicle recycling. These data suggest that inhibition of synaptic vesicle release provides a common response to multiple neurodegenerative disease-linked proteins *in vitro*, and that this common effect is reduced by pre-treatment with VPA or PIA.

$A\beta_{42}$ to binding to synapses was not affected by VPA and PIA – The observations that Aβ accumulated within synapses prior to synapse damage (Lacor et al., 2004) raised the possibility that VPA might prevent synapse damage by reducing the accumulation of Aβ within synapses. To investigate this, we pre-treated neurons with VPA (10 μM) for 1 hour and incubated with 10 nM $A\beta_{42}$, the concentration of $A\beta_{42}$ found in synaptosomes was not significantly altered (2.9 nM $A\beta_{42}$ ± 0.7 compared with 3.2 nM $A\beta_{42}$ ± 0.6, n=9, P=0.5). Similar results were obtained with PIA (10 μM), since it did not significantly affect the binding of $A\beta_{42}$ to synaptosomes (3.1 nM $A\beta_{42}$ ± 0.6 compared with 3.2 nM $A\beta_{42}$ ± 0.6, n=9, P=0.5).

Aβ, αSN and prion-induced hyperactivation of $cPLA_2$ at the synapse was reduced by VPA and PIA - 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) and inhibition of $cPLA_2$ prevented Aβ-induced synapse damage *in vitro* (Bate et al., 2010). In addition, $cPLA_2$ inhibitors ameliorate the cognitive decline seen in a transgenic model of Alzheimer's disease (Sanchez-Mejia et al., 2008). We therefore investigate the activation of $cPLA_2$ in synapses. The addition of Aβ-containing brain extracts, but not Aβ-depleted brain extract, caused a dose-dependent increase in the amounts of activated $cPLA_2$ (Figure 5A). Although VPA, PIA or decaonoic acid (all at 10 μM) did not affect the amounts of $cPLA_2$ protein or activated $cPLA_2$

within synaptosomes (supplementary data, Table 2) the Aβ-induced activation of $cPLA_2$ in synaptosomes was reduced by 1 hour pre-treatment with VPA or PIA, but not with decanoic acid (all at 10 μM). The effects of VPA and PIA upon Aβ-induced activation were dose-dependent and there was no observable difference in their efficacy (Figure 5B). Similarly, pre-treatment of synaptosomes with either VPA or PIA (both at 10 μM) reduced αSN (Figure 5C) and PrP82-146 (Figure 5D)-induced activation of $cPLA_2$. In contrast, pre-treatment with VPA or PIA (both at 10 μM) did not affect the PLAP-induced activation of synaptic $cPLA_2$ (Figure 5E) indicating that these drugs did not have a direct effect upon this enzyme. 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 either VPA or PIA (both at 10 μM) reduced Aβ and αSN-induced PGE_2 production, but had no effect upon PLAP-induced PGE_2 production (Figure 5F). These data suggest that activated $cPLA_2$ activity provides a common response to multiple neurodegenerative disease-linked proteins, and that this common effect is reduced by pre-treatment with VPA or PIA.

The activation of $cPLA_2$ is accompanied by its migration to specific membranes utilizing a Ca^{2+}-dependent lipid binding domain (Nalefski et al., 1994). To investigate $cPLA_2$ localisation in lipid rafts as a cause of enhanced activity, sucrose density gradients generated from synaptosomes in the presence or absence of Aβ (Figure 6A). Lipid rafts were defined by solubility in triton X-100. The addition of Aβ-containing, but not Aβ-depleted brain extract, increased the amounts of $cPLA_2$ within lipid rafts. The addition of Aβ caused a dose-dependent increase in $cPLA_2$ in lipid rafts (Figure 6B). There was a significant correlation between the amounts of $cPLA_2$ in lipid rafts and the amounts of activated $cPLA_2$ in synaptosomes incubated with brain extracts containing between 1 and 0.125 nM $Aβ_{42}$ (Figure 6C). VPA, PIA and decanoic acid alone did not affect the amounts of $cPLA_2$ found within rafts (supplementary data, Table 2). In synaptosomes pre-treated with either VPA or PIA (both at 10 μM), less $cPLA_2$ was found within lipid rafts following the addition of Aβ-containing brain extract (Figure 6D). This data suggest that the Aβ-dependent increase in lipid raft-localised $cPLA_2$ localisation is blocked by the treatment of VPA and PIA.

Aβ-induced increase in synaptic cholesterol is prevented VPA and PIA - The amount of cholesterol in cell membranes is a critical factor involved in both lipid raft formation (Pike, 2004) and in neurodegeneration (Maxfield and Tabas, 2005) and Aβ has been shown to regulate cholesterol homeostasis through an unknown mechanism (Liu et al., 1998). We therefore determined the cholesterol concentrations in synaptosomes following the addition of Aβ-containing brain extract. Increasing concentration of Aβ caused a dose-dependent increase in synaptic cholesterol (Figure 7A) and a corresponding reduction in synaptic cholesterol esters (Figure 7B). There was a significant correlation between the increased concentrations of cholesterol and reduced concentrations of cholesterol esters in synaptosomes incubated with brain extract containing between 1 nM and 0.125 nM $A\beta_{42}$, Pearson's coefficient= -0.931, $P<0.01$ (Figure 7C). These results suggests that Aβ acts through the cholesterol ester cycle, activating cholesterol ester hydrolase enzymes that release biologically active cholesterol into the membrane from stores of cholesterol esters in lipid droplets (Figure 7E). There was also a significant correlation between the concentrations of cholesterol and activated $cPLA_2$ in synaptosomes incubated with brain extract containing between 1 nM and 0.125 nM $A\beta_{42}$, Pearson's coefficient= -0.734, $P<0.01$, (Figure 7D) suggesting that cholesterol concentrations affect the Aβ-induced activation of $cPLA_2$. These data suggest that Aβ acts to alter cholesterol ester hydrolases activity to modify $cPLA_2$ activity by lipid raft localisation.

Since we have shown VPA and PIA reduce Aβ-dependent $cPLA_2$ activation, we then investigated a role for these compounds in cholesterol recycling. While the addition of VPA or PIA or decanoic acid (all at 10 μM) did not affect the amounts of cholesterol in synaptosomes, pre-treatment of synaptosomes with VPA or PIA significantly reduced the Aβ-induced increase in cholesterol in synaptosomes (Figure 8A). Similarly, the Aβ-induced reduction in cholesterol esters was blocked by pre-treatment with VPA or PIA (both at 10 μM; Figure 8B).

Discussion

The discovery of compounds that reduce synapse damage is a rational strategy to reduce clinical symptoms in neurodegenerative diseases including prion diseases, Alzheimer's disease and Parkinson's disease. In this study we demonstrate that VPA and PIA protected cultured neurons against synapse damage induced by the neurotoxic peptides Aβ, PrP82-146 and αSN. Neuroprotection was associated with the regulation of cholesterol and inhibition of $cPLA_2$, hyperactivation of which leads to synapse degeneration and memory defects (Bate et al., 2010; Sanchez-Mejia et al., 2008). This study used 2 models of synapse damage; firstly the loss of synaptic proteins from cultured neurons and secondly the inhibition of fluorescent FM1-43 uptake into synaptosomes as an indicator of the synapse vesicle recycling that is necessary for normal neurotransmission. The loss of synaptic proteins from neurons indicated synapse degeneration and the disruption in synaptic vesicle recycling demonstrated synapse dysfunction. Both these effects were apparent at concentrations of neurotoxic peptides that did not cause neuronal death. In this respect we have sought to replicate the early stages of disease where there is significant synapse dysfunction/damage but without the extensive loss of neurons that occurs during the latter stages of disease.

Our date suggests that VPA may attenuate the Aβ-induced synapse damage that underlies the dementia observed in Alzheimer's disease patients. Similarly, VPA protected neurons against synapse damage induced by αSN, aggregates of which accumulate in synapses during Parkinson's disease and Lewy body dementia (Kramer and Schulz-Schaeffer, 2007) and the prion-derived peptide PrP82-146. We demonstrate a mechanism for this neuroprotective effect through inhibiting hyperactivation of $cPLA_2$ by these neurotoxic peptides. Consistent with this mechanism, VPA has been reported to show PLA_2 inhibitory-like activity (Bosetti et al., 2003; Elphick et al., 2012; Rapoport and Bosetti, 2002), in addition to inhibiting phosphoinositide turnover (Chang et al., 2012) and histone deactylase activity (Phiel et al., 2001). PIA is thought to have greater therapeutic potential as it does not inhibit histone deacteylase (Eyal et al., 2005), an activity associated with teratogenic effects (Jentink et al., 2010). Although a direct mechanism of action

has been recently reported for PIA, through an inhibition of acyl-CoA synthetase (Modi et al., 2013), this effect occurs with a Ki of 11.4 mM, that is around 1000-fold higher than effective concentrations reported here. PIA has also been shown to strongly reduce fatty acid release (Elphick et al., 2012) consistent with an indirect effect on $cPLA_2$ activity shown here. Decanoic acid was used in this study, since like VPA, it has also been reported to inhibit phosphoinositide turnover in relation to seizure control (Chang et al., 2012), although again at higher concentrations than used in this study, thus it appears that VPA and PIA exert different effects on neuroprotection that decanoic acid at these concentrations. Neither VPA nor PIA protected cultured neurons against synapse damage triggered by PLAP indicating that VPA and PIA affect specific pathways that are activated by neurotoxic peptides.

VPA has been reported to reduce Aβ production (Qing et al., 2008b; Su et al., 2004) and attenuate neuronal loss in a murine transgenic model of Alzheimer's disease (Long et al., 2013). Our study showed that neither VPA nor PIA significantly altered the amounts of $A\beta_{42}$ that bound to synapses. The observation that Aβ can accumulate within synapses without causing synapse damage indicates that synapse damage occurs via activation of specific pathways, rather than a direct effect of Aβ itself. Prior studies had demonstrated the importance of $cPLA_2$ in Alzheimer's disease, pharmacological inhibition of $cPLA_2$ protected neurons against Aβ-induced synapse damage (Bate et al., 2010) and inhibited cognitive impairment in a murine model of Alzheimer's disease (Sanchez-Mejia et al., 2008). Others have reported that VPA and PIA alter arachidonic acid metabolism (Bazinet et al., 2006) and here we showed that VPA and PIA, but not decanoic acid, reduced the activation of synaptic $cPLA_2$ by Aβ, PrP82-146 or αSN. It was notable that the concentrations of VPA and PIA required to inhibit Aβ-induced activation of synaptic $cPLA_2$ were similar to those that reduced Aβ-induced synapse damage, observation supporting the direct role of activated $cPLA_2$ in Aβ-induced synaptotoxicity. The activation of $cPLA_2$ is critical for the formation of bioactive lipids including prostaglandins and PAF. The neurotoxic peptides used in this study activates $cPLA_2$ leading to the production of PGE_2 which caused synapse damage (Bate et al., 2010) and are raised in the cerebrospinal fluid of patients with probable Alzheimer's disease (Montine et al., 1999).

Neither VPA nor PIA affected the activation of $cPLA_2$ by PLAP suggesting that they did not have a direct effect upon the enzyme. The activation of $cPLA_2$ is accompanied by its migration to specific membranes utilizing a Ca^{2+}-dependent lipid-binding domain (Nalefski et al., 1994). Here we show that Aβ causes $cPLA_2$ to be targeted to lipid rafts and that in synaptosomes there was a significant correlation between the % of $cPLA_2$ in rafts and activation of $cPLA_2$ following the addition of Aβ. The recruitment of signalling proteins to specific compartments is an emerging concept in the regulation of cell activation. The observations that Aβ is found within lipid rafts (Williamson et al., 2008) and the Aβ-induced activation of $cPLA_2$ is cholesterol sensitive (Bate and Williams, 2007) suggests that the formation of specific lipid rafts is necessary for Aβ-induced activation of $cPLA_2$ and synapse degeneration. Critically, pre-treatment with VPA or PIA reduced the Aβ-induced translocation of $cPLA_2$ to rafts.

It should be noted that $cPLA_2$ and the products of its activation including PAF and prostaglandins are involve in the normal function of the synapse. Both PGE_2 and PAF have both been implicated in the induction of long-term potentiation and memory formation (Chen and Bazan, 2005). Our contention is that it is Aβ-induced aberrant activation of $cPLA_2$ that leads to synapse degeneration. Therefore, the inhibition of $cPLA_2$ by VPA and PIA may be beneficial under these conditions. However, long term VPA treatment is associated with impairment of hippocampal synaptic plasticity (Sgobio et al., 2010) and *in vitro* 1 mM VPA reduced long term potentiation in hippocampal slices (Chang et al., 2010) indicating that VPA and PIA may also reduce activation of $cPLA_2$ in normal synapses.

Aβ has been shown to affect cholesterol homeostasis and in these neurons Aβ increased cholesterol concentrations in synaptosomes; an effect consistent with observations of increased concentrations of cholesterol in Aβ positive synapses in the cortex of Alzheimer's patients (Gylys et al., 2007). The Aβ-induced increase in cholesterol may be significant in that cholesterol an essential role in their formation and function of lipid rafts (Pike, 2004) and there was a significant correlation between the increase in cholesterol

and activated $cPLA_2$ in response to Aβ. Notably, VPA and PIA did not affect cholesterol concentrations in control synaptosomes but both reduced the Aβ-induced increase in cholesterol. Our studies showed that the Aβ-induced increase in synaptic cholesterol was accompanied by a reduction in cholesterol esters indicating that Aβ activated cholesterol ester hydrolase. The observation that VPA and PIA prevented the Aβ-induced reduction in concentrations of cholesterol esters suggests that these drugs inhibit cholesterol ester hydrolase.

Conclusion

In summary we report that VPA and PIA protected cultured neurons against the synapse damage that was induced by Aβ, αSN or PrP82-146, neurotoxic peptides associated with disease progression in Alzheimer's, Parkinson's and prion disease. Our results are consistent with the hypothesis that VPA and PIA inhibit the peptide-induced activation of $cPLA_2$, hyperactivation of which leads to synapse damage. These drugs do not appear to have a direct effect upon $cPLA_2$, rather they affected the membrane micro-environment in which peptides activate $cPLA_2$, inhibiting the release of cholesterol that is required for a stable signalling platform. These studies suggest that VPA and PIA may be able to reduce synapse damage and ameliorate pathology in Alzheimer's, Parkinson's and prion diseases.

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Authors' contributions – CB: conception and design, data collection and analysis, manuscript writing and revision. RW: conception and design, manuscript writing and revision. Both authors read and approved the final manuscript.

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Figure Legends

Figure 1. Soluble Aβ triggers synapse damage in neurons - (A) Immunoblot showing Aβ monomers (M), dimers (D) and trimers (T) in brain extract (1) and Aβ-depleted brain extract (2). (B) Aβ monomers (M), dimers (D) and trimers (T) eluted from a Superdex 75 PC column (separates proteins ranging from 3 kDa to 70 kDa) loaded with a soluble brain extract. Values are means of duplicates. The amounts of synaptophysin (C) and CSP (D) in neurons incubated with brain extracts (●), Aβ-depleted brain extracts (▲) or mock-depleted brain extracts (Δ). Values are means ± SD from triplicate experiments performed 4

times, n=12. (E) Immunoblots showing the amounts of synaptophysin, CSP synapsin-1, VAMP-1 and caveolin in neurons incubated with brain extract as shown.

Figure 2 - Synapse damage induced by Aβ is reduced by VPA and PIA - The amounts of (A) synaptophysin and (B) CSP in neurons pre-treated with control medium (●), 10 μM VPA (○), 10 μM PIA (□) or 10 μM decanoic acid (■) and incubated with brain extracts containing $A\beta_{42}$ as shown. Values are means ± SD from triplicate experiments performed 4 times, n=12. (C) The amounts of synaptophysin in neurons pre-treated with VPA (●), PIA (□) or decanoic acid (■) and incubated with brain extract containing 2 nM $A\beta_{42}$. Values are means ± SD from triplicate experiments performed twice, n=6.

Figure 3. Synapse damage induced by αSN and PrP82-146 is reduced by VPA and PIA – The amounts of synaptophysin (A) and CSP (B) in neurons pre-treated with control medium (●), 10 μM VPA (○), 10 μM PIA (□) or 10 μM decanoic acid (■) and incubated with αSN as shown. The amounts of synaptophysin (C) and CSP (D) in neurons pre-treated with control medium (●), 10 μM VPA (○), 10 μM PIA (□) or 10 μM decanoic acid (■) and incubated with PrP82-146 as shown. The amounts of synaptophysin (E) and CSP (F) in neurons pre-treated with control medium (●), 10 μM VPA (○), 10 μM PIA (□) or 10 μM decanoic acid (■) and incubated with PLAP as shown. All values are means ± SD from triplicate experiment performed 4 times, n=12.

Figure 4. Synaptic vesicle recycling is inhibited by Aβ, αSN and PrP82-146 and is rescued by VPA and PIA – (A) The amounts of FM1-43 in synaptosomes pre-treated with control medium (●), 10 μM VPA (○), 10 μM PIA (□) or 10 μM decanoic acid (■) and pulsed with ionomycin as shown (●). Values are mean % fluorescence (100%=maximum fluorescence in synaptosomes). (B) The amounts of FM1-43 in synaptosomes incubated with brain extract (●), Aβ-depleted brain extract (▲) or mock-depleted brain extract (Δ) and pulsed with ionomycin. The amounts of FM1-43 in synaptosomes pre-treated with control medium (●), 10 μM VPA (○), 10 μM PIA (□) or 10 μM decanoic acid (■), incubated with brain extract

containing $A\beta_{42}$ as shown (C), PrP82-146 (D) or αSN (E) and pulsed with ionomycin. All values are means ± SD from triplicate experiments performed 3 times, n=9.

Figure 5. Induced activation of synaptic $cPLA_2$ by Aβ, αSN and PrP82-146 is reduced by VPA and PIA (A) The amounts of activated $cPLA_2$ in synaptosomes pre-treated with control medium (●), 10 μM VPA (○), 10 μM PIA (□) or 10 μM decanoic acid (■) and incubated with brain extract containing $A\beta_{42}$ as shown. (B) The amount of activated $cPLA_2$ in synaptosomes pre-treated with VPA (○), PIA (□) or decanoic acid (■) and incubated with brain extract containing 2 nM $A\beta_{42}$. The amounts of activated $cPLA_2$ in synaptosomes pre-treated with control medium (●), 10 μM VPA (○), 10 μM PIA (□) or 10 μM decanoic acid (■) and incubated with (C) αSN or (D) PrP82-146 as shown. (E) The amounts of activated $cPLA_2$ in synaptosomes pre-treated with control medium (●), 10 μM VPA (○), 10 μM PIA (□) or 10 μM decanoic acid (■) and incubated PLAP as shown. All values are means ± SD, from triplicate experiments performed 3 times, n=9. (F) The concentrations of PGE_2 in synaptosomes pre-treated with control medium (■), 10 μM VPA (□) or 10 μM decanoic acid (striped bar) and incubated with brain extract containing 2 nM $A\beta_{42}$, 500 nM αSN or 500 nM PLAP. Values are means ± SD, n=6. *=PGE_2 significantly less than in control synaptosomes incubated with peptides.

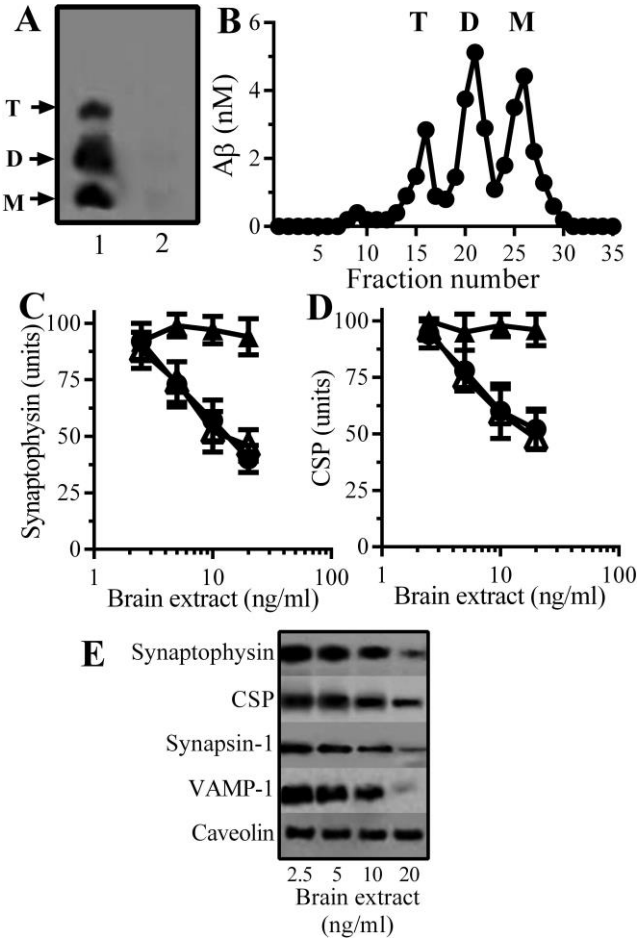
Figure 6. Aβ-induced translocation of $cPLA_2$ into rafts is reduced by VPA and PIA – (A) The amount of $cPLA_2$ in fractions from control synaptosomes (○) or synaptosomes incubated with brain extract containing 1 nM $A\beta_{42}$ (●). (B) The amounts of $cPLA_2$ in lipid rafts derived from synaptosomes incubated with control medium (□) or brain extract containing $A\beta_{42}$ as shown. (C) There was a significant correlation between the amounts of raft $cPLA_2$ and activated $cPLA_2$ in synaptosomes incubated with brain extract containing $A\beta_{42}$ (1 nM to 0.6 nM), Pearson's coefficient=0.927, $P<0.01$. (D) The amounts of $cPLA_2$ in lipid rafts derived from synaptosomes pre-treated with control medium (□), 10 μM VPA (■), 10 μM PIA (striped bar) or 10 μM decanoic acid (DA) (hatched bar) and incubated with brain extract containing 1 nM $A\beta_{42}$.

All values are means ± SD, from triplicate experiments performed 3 times, n=9. *=amounts of raft $cPLA_2$ significantly less than in control synaptosomes incubated with Aβ.

Figure 7. Aβ-induces increased synaptic cholesterol and reduced cholesterol esters – The concentrations of cholesterol (A) and cholesterol esters (B) in synaptosomes incubated with control medium (□) or brain extract containing $A\beta_{42}$ as shown (■). Values are means ± SD from triplicate experiments performed 4 times (n=12). *=cholesterol significantly higher than in control synaptosomes. **=cholesterol esters significantly lower than in control synaptosomes. (C) There was a significant inverse correlation between the concentrations of cholesterol and cholesterol esters in synaptosomes incubated with brain extract containing $A\beta_{42}$ (1 nM to .125 nM), Pearson's coefficient= -0.931, $P<0.01$. (D) There was a significant correlation between the concentrations of cholesterol and amounts of activated $cPLA_2$ in synaptosomes incubated with brain extract containing $A\beta_{42}$ (1 nM to .125 nM), Pearson's coefficient= -0.734, $P<0.01$. (E) Schematic showing the cholesterol ester cycle and the proposed release of synaptic cholesterol via Aβ-induced activation of cholesterol ester hydrolases (CEH).

Figure 8. Aβ-induced changes in synaptic cholesterol is reduced by VPA and PIA – The concentrations of cholesterol (A) and cholesterol esters (B) in synaptosomes pre-treated with control medium, 10 μM VPA, 10 μM PIA or 10 μM decanoic acid (DA) and incubated with control medium (□) or brain extract containing 1 nM $A\beta_{42}$ (■). *=cholesterol significantly lower than in control synaptosomes incubated with Aβ. **=cholesterol esters significantly higher than in control synaptosomes incubated with Aβ.

Figure 1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Fraction number
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Synaptophysin (units)
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D
CSP (units)
Brain extract (ng/ml)
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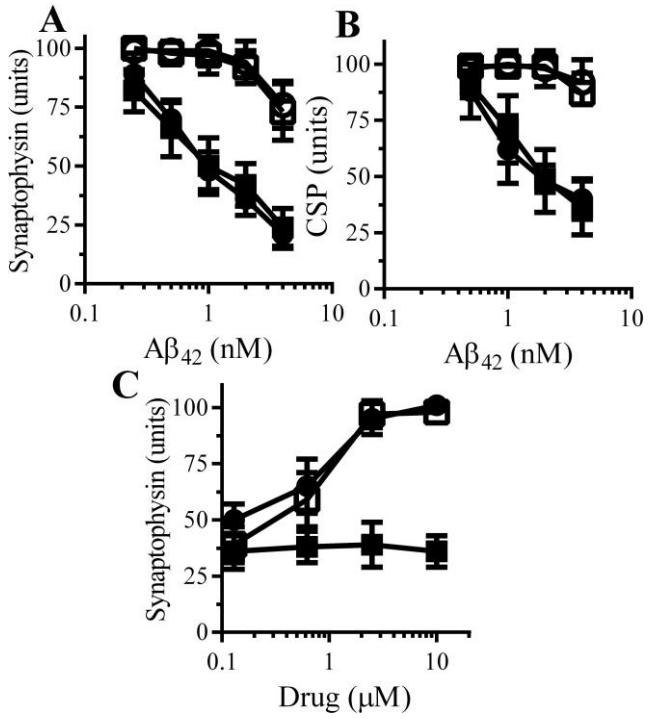
Brain extract
(ng/ml)

Figure 2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Drug (μM)

Figure 3

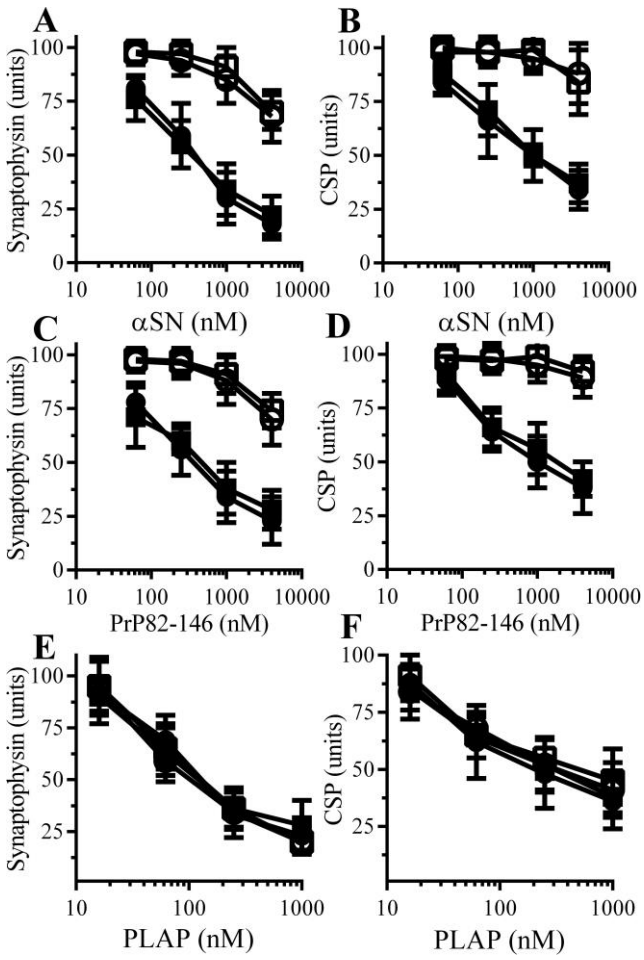
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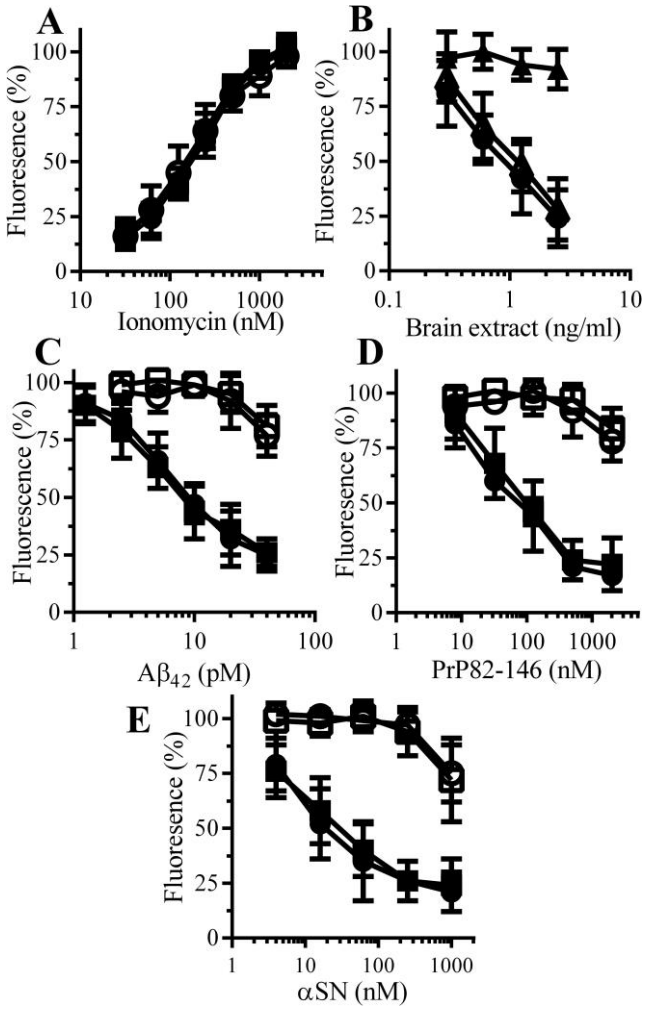
Figure 4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Fluorescence (%)
Brain extract (ng/ml)
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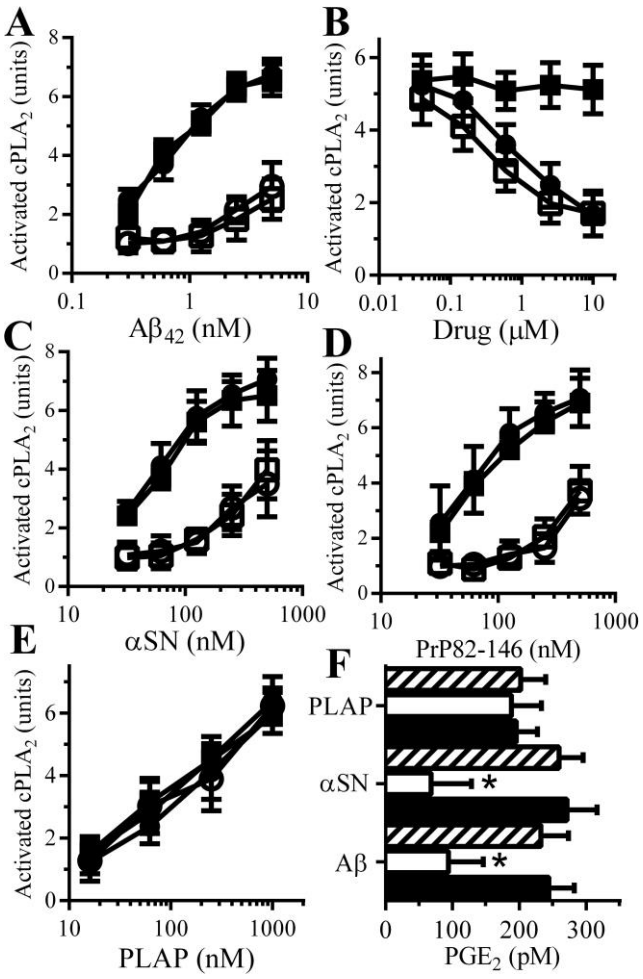
Fluorescence (%)
αSN (nM)

Figure 5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Drug (μM)
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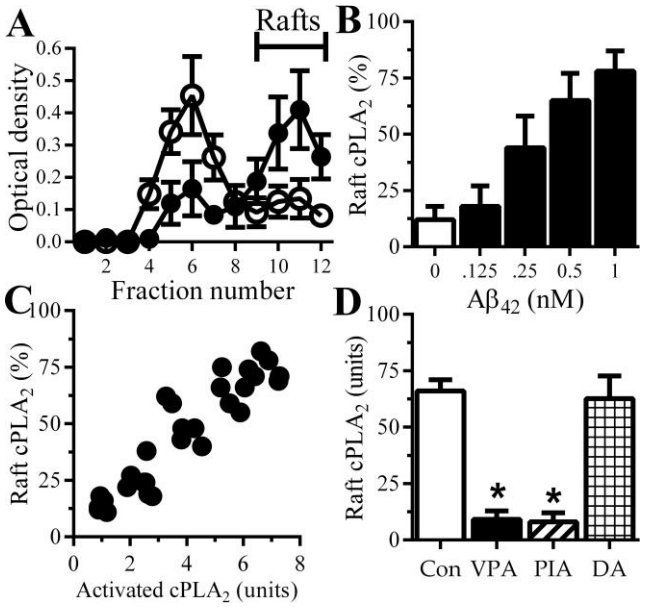
Aβ
PGE2 (pM)

Figure 6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Rafts
Optical density
Fraction number
B
Raft cPLA2 (%)
Aβ42 (nM)
C
Raft cPLA2 (%)
Activated cPLA2 (units)
D
Raft cPLA2 (units)
Con VPA PIA DA

Figure 7

Figure 8