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RESEARCH ARTICLE

The cholesterol ester cycle regulates signalling complexes and synapse damage caused by amyloid-β

Ewan West, Craig Osborne and Clive Bate*

ABSTRACT

Cholesterol is required for the formation and function of some signalling platforms. In synaptosomes, amyloid-β (Aβ) oligomers, the causative agent in Alzheimer's disease, bind to cellular prion proteins (PrP^C) resulting in increased cholesterol concentrations, translocation of cytoplasmic phospholipase A_2 ($cPLA_2$, also known as PLA2G4A) to lipid rafts, and activation of $cPLA_2$. The formation of Aβ-PrP^C complexes is controlled by the cholesterol ester cycle. In this study, Aβ activated cholesterol ester hydrolases, which released cholesterol from stores of cholesterol esters and stabilised Aβ-PrP^C complexes, resulting in activated $cPLA_2$. Conversely, cholesterol esterification reduced cholesterol concentrations causing the dispersal of Aβ-PrP^C complexes. In cultured neurons, the cholesterol ester cycle regulated Aβ-induced synapse damage; cholesterol ester hydrolase inhibitors protected neurons, while inhibition of cholesterol esterification significantly increased Aβ-induced synapse damage. An understanding of the molecular mechanisms involved in the dispersal of signalling complexes is important as failure to deactivate signalling pathways can lead to pathology. This study demonstrates that esterification of cholesterol is a key factor in the dispersal of Aβ-induced signalling platforms involved in the activation of $cPLA_2$ and synapse degeneration.

KEY WORDS: Amyloid-β, Cholesterol, Cholesterol ester hydrolases, Phospholipase A_2, Prion, Rafts, Synapse

INTRODUCTION

The cellular prion protein (PrP^C) gained notoriety for its role in the transmissible spongiform encephalopathies after undergoing transformation to the disease-associated isoform (PrP^{Sc}). While the normal role of PrP^C remains unclear, reports that PrP^C is concentrated at synapses (Herms et al., 1999), and that transgenic mice in which the gene for PrP had been knocked-out [$Prnp^{(0/0)}$] showed synaptic and memory deficits (Maglio et al., 2006), suggest that it plays a role in neurotransmission. PrP^C is attached to cell membranes via a glycosylphosphatidylinositol (GPI) anchor (Stahl et al., 1987), which targets the protein to specific membrane micro-domains called lipid rafts (Taraboulos et al., 1995). Many lipid rafts are enriched with signalling molecules and act as platforms in which GPI anchors interact with signalling proteins (Sharma et al., 2004; Suzuki et al., 2007). PrP^C is associated with numerous cell signalling pathways, including those of the tyrosine kinase Fyn (Mouillet-Richard et al., 2000), protein kinase A (Chiarini et al., 2002) and cytoplasmic phospholipase A_2 ($cPLA_2$, also known as PLA2G4A) (Bate et al., 2010). The formation of signalling complexes can be triggered by the cross-linkage of lipid raft components, including the oligomerization of GPI-anchored proteins (Hammond et al., 2005; Lingwood et al., 2008). PrP^C acts as a scaffold protein that organises signalling complexes (Linden et al., 2012), and in neurons the clustering of specific GPIs attached to PrP^C caused aberrant cell signalling and synapse degeneration (Bate and Williams, 2012). Because GPI-anchored proteins are surrounded by a shell of membrane lipids (Anderson and Jacobson, 2002), the selective associative properties of cholesterol, sphingolipids and GPI-anchored proteins are capable of altering raft composition and function (Lingwood and Simons, 2010). The aggregation of GPIs attached to PrP^C is thought to alter the composition of the underlying cell membrane, leading to cell activation in a process similar to that reported in T cell signalling (Chen et al., 2006; Suzuki et al., 2007).

Synaptic abnormalities are caused by aggregated PrP^C (Chiesa et al., 2008) and by cross-linkage of PrP^C with monoclonal antibodies (mAbs) (Solforosi et al., 2004). More recently, PrP^C was identified as a receptor for amyloid-β (Aβ) oligomers (it is predominantly $Aβ_{42}$ and $Aβ_{40}$ peptides that are associated with disease) (Laurén et al., 2009) that are responsible for the synapse degeneration and cognitive decline in patients with Alzheimer's disease (AD) (Selkoe, 2002; Shankar et al., 2008). Cross-linkage of PrP^C by Aβ oligomers forms a signalling complex containing activated $cPLA_2$ (Bate and Williams, 2011) and leads to the production of platelet-activating factor (PAF) and prostaglandins (PGs). The observations that $cPLA_2$ is highly enriched at synapses (Moskowitz et al., 1983), and that PAF and PGs affect synaptic plasticity and memory formation (Chen and Bazan, 2005; Koch et al., 2010), suggest that controlled activation of this enzyme is a normal aspect of synapse function. However, aberrant activation of $cPLA_2$ is associated with synapse degeneration and clinical symptoms as the concentrations of PGE_2 and PAF are increased in the brains of AD patients when compared with non-demented controls (Montine et al., 1999; Ryan et al., 2009).

Cholesterol is highly enriched in synaptic membranes and the fine tuning of cholesterol dynamics is thought to underlie synapse plasticity and hence memory (Linetti et al., 2010). Given that the formation and function of lipid rafts depends upon cholesterol concentrations (Rajendran and Simons, 2005), it follows that fluctuations in cholesterol concentrations alter the functions of lipid rafts. The key finding in this study was that the addition of soluble Aβ oligomers, highly toxic forms of Aβ (Yang et al., 2017), increased synaptic cholesterol concentrations. Soluble Aβ oligomers isolated from brain extracts had effects at picomolar concentrations, concentrations similar to those in cerebrospinal fluid (Mc Donald et al., 2010; McLean et al., 1999). The increase in cholesterol was accompanied by a corresponding decrease in cholesterol esters and was blocked by selective cholesterol ester

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hydrolase (CEH) inhibitors. Pre-treatment of synaptosomes with CEH inhibitors also reduced the formation of Aβ-PrP^C complexes, the Aβ-induced translocation $cPLA_2$ to lipid rafts and activation of $cPLA_2$. In cultured neurons, CEH inhibitors reduced Aβ-induced synapse degeneration.

The dissociation of signalling platforms is thought to be a physiological process that limits the intensity of cell signalling. Consequently, conditions that prevent the dissociation of signalling platforms may lead to sustained activation, cell disruption and pathology. This study shows that in synaptosomes, esterification of cholesterol dispersed Aβ-PrP^C complexes and reduced cell signalling, and in neuronal cultures it reduced Aβ-induced synapse damage. Our results are consistent with the hypothesis that the esterification of cholesterol is a key component mediating the dissociation of Aβ-induced signalling platforms involved in synapse damage.

RESULTS

Soluble Aβ increases cholesterol concentrations in synapses

As soluble Aβ oligomers are too small to be identified via electron microscopy (Walsh et al., 2002), they were characterised using gel electrophoresis. Brain extracts contained Aβ monomers, dimers and trimers that were removed following immunodepletion with mAb 4G8 (reactive with epitopes 17–24 of Aβ) (Fig. 1A). The addition of brain extracts containing 1 nM $Aβ_{42}$ significantly increased the concentrations of cholesterol within synaptosomes (Fig. 1B). Immunodepletion with mAb 4G8 reduced the concentrations of $Aβ_{42}$ (1±0.07 nM compared with 0.03±0.015 nM, n=9, P<0.01) and $Aβ_{40}$ (4.36±0.22 nM compared with 0.24±0.07 nM, n=9, P<0.01) in brain extracts, while immunodepletion with mAb 3F4 (reactive with human prion proteins, mock-depletion) had no significant effect on either $Aβ_{42}$ (1±0.07 nM compared with 0.98 ±0.08 nM, n=9, P=0.38) or $Aβ_{40}$ (4.36±0.22 nM compared with 4.15±0.28 nM, n=9, P=0.45). Aβ-depleted brain extracts did not alter synaptic cholesterol concentrations, indicating that the increase in cholesterol was triggered by Aβ. The addition of brain extract containing 1 nM $Aβ_{42}$ to synaptosomes from neurons derived from Prnp knockout$^{(0/0)}$ mice did not significantly alter cholesterol concentrations (0.83±0.07 µM compared with 0.87±0.07 µM, P=0.23, n=9).

The addition of Aβ oligomers caused a dose-dependent increase in synaptic cholesterol concentrations, whereas Aβ monomers had no significant effect (Fig. 1C). Pre-treatment with 1 µM squalestatin, a squalene synthetase inhibitor (Baxter et al., 1992), did not significantly alter the Aβ-induced increase in synaptic cholesterol concentrations (Fig. 1D), indicating that Aβ did not stimulate cholesterol synthesis. The addition of brain extracts to synaptosomes reduced the concentrations of cholesterol esters, and there was a significant inverse correlation between the concentrations of cholesterol and cholesterol esters in synaptosomes incubated with brain extract containing 0.125–1 nM $Aβ_{42}$ (Fig. 1E). However, the effects of Aβ on synaptic cholesterol were transient; changes in cholesterol/cholesterol ester concentrations were seen 1 and 2 h after the addition of 1 nM $Aβ_{42}$ but had returned to normal after 4 h (Fig. 1F). Collectively, these findings suggest that the Aβ-induced increase in cholesterol was provided by the hydrolysis of cholesterol esters (Fig. 1G).

The cholesterol ester cycle controls the Aβ-induced increase in cholesterol

This hypothesis was tested using the CEH inhibitors diethylumbelliferyl phosphate (DEUP) (Gocze and Freeman,

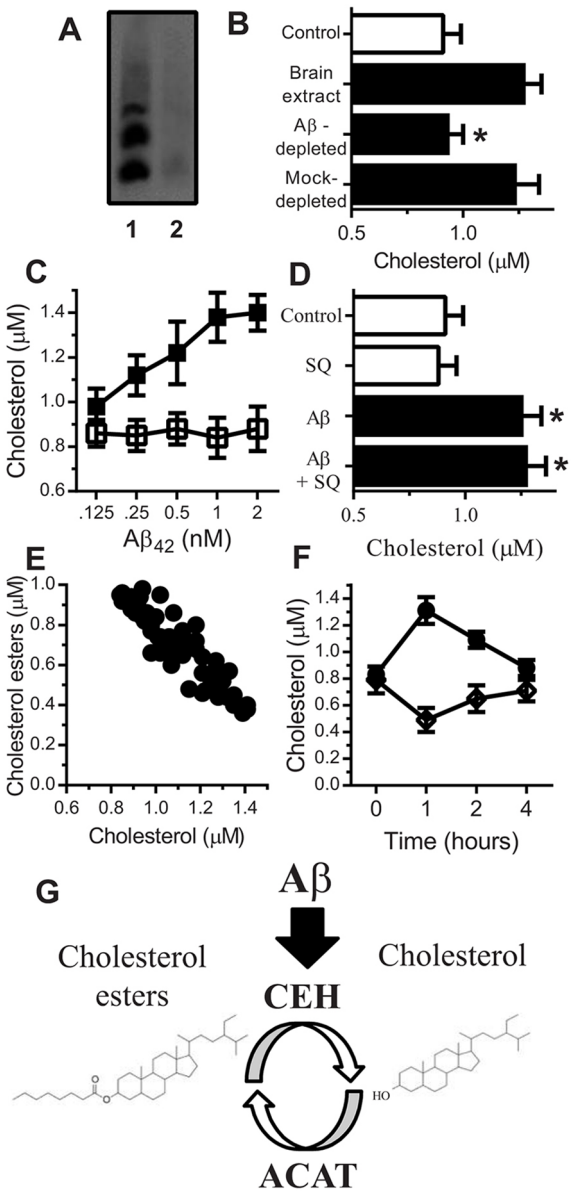

Fig. 1. The Aβ-induced increase in cholesterol is mediated by hydrolysis of cholesterol esters. (A) Immunoblot showing forms of Aβ in brain extract (1) and in Aβ-depleted brain extract (2). (B) The concentrations of cholesterol in synaptosomes incubated with control medium, brain extracts, and Aβ-depleted or mock-depleted brain extracts. Values are mean±s.d. from triplicate experiments performed three times (n=9). The asterisk indicates cholesterol concentrations significantly lower than in synaptosomes incubated with brain extract (t-test, P<0.01). (C) The concentrations of cholesterol in synaptosomes incubated with oligomer (■) or monomer (□) preparations containing $Aβ_{42}$ as shown. Values are mean±s.d. from triplicate experiments performed three times (n=9). (D) The concentrations of cholesterol in synaptosomes treated with control medium or 1 µM squalestatin (SQ) (□) or pre-treated with control medium or 1 µM SQ and incubated with brain extract containing 1 nM $Aβ_{42}$ (■). Values are mean±s.d. from triplicate experiments performed three times; n=9. The asterisks indicate concentrations of cholesterol significantly higher than in control synaptosomes (t-test, P<0.01). (E) There was a significant inverse correlation between the concentrations of cholesterol and cholesterol esters in synaptosomes incubated with brain extract containing $Aβ_{42}$ (0.125–1 nM; Pearson's coefficient=−0.93, P<0.01). (F) The concentrations of cholesterol (●) and cholesterol esters (◊) in synaptosomes incubated with brain extract containing 1 nM $Aβ_{42}$ for the indicated time periods. Values are mean±s.d. from triplicate experiments performed three times; n=9. (G) Schematic of the proposed control of synaptic cholesterol concentrations via Aβ-induced activation of CEH and ACAT.

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1992) and cholesteryl N-(2-dimethylaminoethyl) carbamate (Hosie et al., 1987). Pre-treatment of synaptosomes with either 20 µM DEUP or 5 µM cholesteryl N-(2-dimethylaminoethyl) carbamate inhibited both the Aβ-induced increase in cholesterol concentrations (Fig. 2A) and the Aβ-induced reduction in cholesterol esters (Fig. 2B). These drugs did not affect the concentrations of cholesterol or cholesterol esters in control synaptosomes.

To determine whether acetyl-coenzyme A acetyltransferases (ACAT), an enzyme that esterifies cholesterol in cell membranes (Chang et al., 2006), was involved in regulating Aβ-induced changes in membrane cholesterol concentrations, neurons were treated with the ACAT inhibitors (TMP-153) (Sugiyama et al., 1995) or YIC-C8-434 (Kaneko et al., 2001). First, the efficacy of ACAT inhibitors was determined by incubating neurons with squalene. The addition of 5 µM squalene, which was converted to cholesterol and subsequently esterified by ACAT, resulted in high concentrations of cholesterol esters in synaptosomes (Fig. 2C). Pre-treatment of neurons with either TMP-153 or YIC-C8-434 reduced the squalene-induced increase in cholesterol esters, indicating inhibition of ACAT (Fig. 2D). At concentrations of 100 nM, both TMP-153 and YIC-C8-434 fully blocked the squalene-induced increase in cholesterol ester concentrations. Subsequently, pre-treatment of synaptosomes with 100 nM TMP-153 or YIC-C8-434 significantly enhanced the Aβ-induced increase in cholesterol concentrations (Fig. 2E), providing evidence that ACAT reverses the Aβ-induced increase in cholesterol.

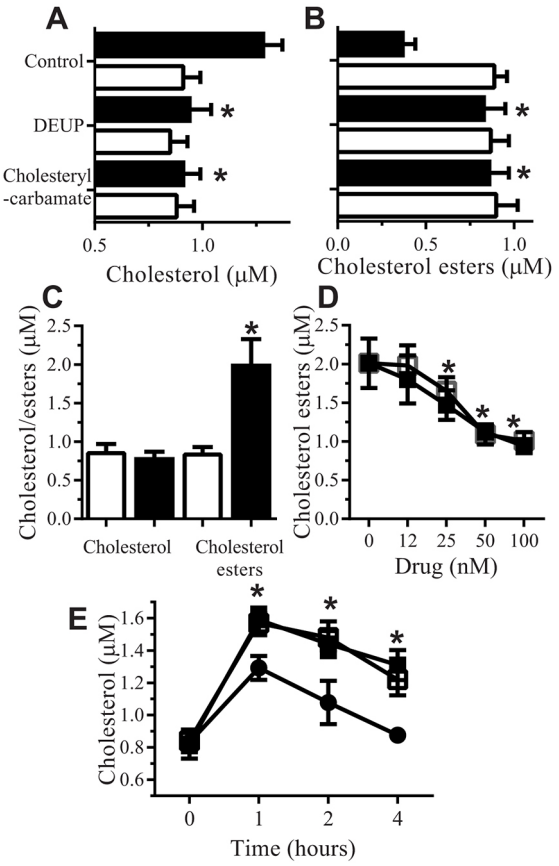

Fig. 2. CEH inhibitors block the Aβ-induced increase in cholesterol. (A,B) The concentrations of cholesterol (A) and cholesterol esters (B) in synaptosomes pre-treated with CEH inhibitors, 20 µM DEUP or 5 µM cholesteryl N-(2-dimethylaminoethyl) carbamate, and incubated with control medium (□) or brain extract containing 1 nM $Aβ_{42}$ (■). Values are mean±s.d. from triplicate experiments performed three times; *n*=9. The asterisks indicate concentrations of cholesterol/cholesterol esters significantly different to control synaptosomes incubated with Aβ (*t*-test, *P*<0.01). (C) The concentrations of cholesterol and cholesterol esters in synaptosomes derived from neurons treated with control medium (□) or 5 µM squalene (■). Values are mean±s.d. from triplicate experiments performed three times; *n*=9. The asterisk indicates concentrations of cholesterol esters significantly higher than in control synaptosomes (*t*-test, *P*<0.01). (D) The concentrations of cholesterol esters in synaptosomes from neurons pre-treated with ACAT inhibitors, TMP-153 (■) or YIC-C8-434 (□), and incubated with 5 µM squalene. Values are mean±s.d. from triplicate experiments performed three times; *n*=9. The asterisks indicate concentrations of cholesterol esters significantly lower than in control synaptosomes incubated with squalene (*t*-test, *P*<0.01). (E) The concentrations of cholesterol in synaptosomes pre-treated with control medium (●), 100 nM TMP-153 (■) or 100 nM YIC-C8-434 (□) and incubated with brain extract containing 1 nM $Aβ_{42}$ for the indicated time periods. Values are mean ±s.d. from triplicate experiments performed three times; *n*=9. The asterisks indicate cholesterol concentrations significantly higher than in control synaptosomes incubated with Aβ, (*t*-test, *P*<0.01).

The cholesterol ester cycle controls PrP^C-Aβ complexes

Cholesterol, but not cholesterol esters, affects membrane structure and function. For example, cholesterol concentrations can affect the expression of PrP^C (Gilch et al., 2006), which acts a receptor for $Aβ_{42}$ (Laurén et al., 2009). The addition of brain extract to synaptosomes caused the formation of PrP^C-Aβ complexes (Fig. 3A). There was a significant correlation between the

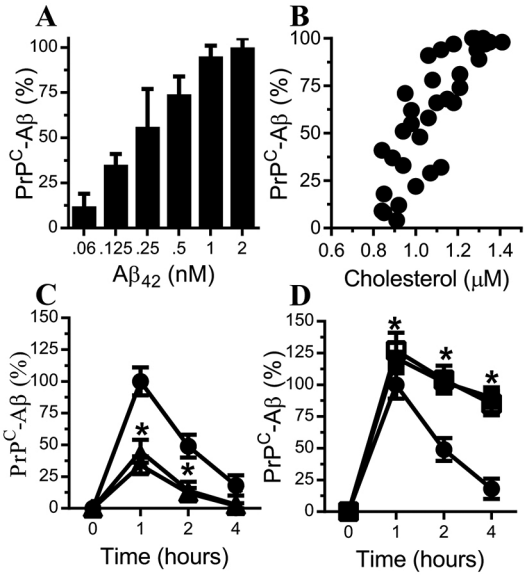

Fig. 3. The cholesterol ester cycle regulates PrP^C-Aβ complexes. (A) The amounts of PrP^C-Aβ complexes in synaptosomes incubated with brain extract containing $Aβ_{42}$ as shown. Values are mean±s.d. from triplicate experiments performed three times; *n*=9. (B) There was a significant correlation between the amounts of PrP^C-Aβ complexes and concentrations of cholesterol following the addition of brain extract containing $Aβ_{42}$ (0.06–1 nM, Pearson's coefficient=0.85, *P*<0.01). (C) The amounts of PrP^C-Aβ complexes in synaptosomes pre-treated with control medium (●), 20 µM DEUP (▲) or 5 µM cholesteryl N-(2-dimethylaminoethyl) carbamate (△) and incubated with brain extract containing 1 nM $Aβ_{42}$ for the indicated time periods. Values are mean ±s.d. from triplicate experiments performed three times; *n*=9. The asterisks indicate significantly less PrP^C-Aβ complexes than in control synaptosomes incubated with Aβ (*t*-test, *P*<0.01). (D) The amounts of PrP^C-Aβ complexes in synaptosomes pre-treated with control medium (●), 100 nM TMP-153 (■) or 100 nM YIC-C8-434 (□) and incubated with brain extract containing 1 nM $Aβ_{42}$ for the indicated time periods. Values are mean±s.d. from triplicate experiments performed three times; *n*=9. The asterisks indicate significantly more PrP^C-Aβ complexes than in control synaptosomes incubated with Aβ (*t*-test, *P*<0.01).

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amounts of PrP^C-Aβ complexes and cholesterol concentrations in synaptosomes incubated with brain extracts containing between 0.06 and 1 nM $Aβ_{42}$ (Pearson's coefficient= 0.85, $P<0.01$) (Fig. 3B). As both PrP^C (Naslavsky et al., 1997) and Aβ (Kawarabayashi et al., 2004; Williamson et al., 2008) are found within lipid rafts, the formation of which is cholesterol sensitive (Schroeder et al., 1994), we hypothesized that cross-linkage of PrP^C by Aβ oligomers stimulated the release of cholesterol that stabilised PrP^C-Aβ complexes. A time course study demonstrated that PrP^C-Aβ complexes were transient (Fig. 3C), as was the Aβ-induced increase in cholesterol (Fig. 1F), suggesting that PrP^C-Aβ complexes and cholesterol concentrations were linked. Pre-treatment of synaptosomes with CEH inhibitors [20 µM DEUP or 5 µM cholesteryl N-(2-dimethylaminoethyl) carbamate] reduced the formation of PrP^C-Aβ complexes (Fig. 3C). By contrast, pre-treatment of synaptosomes with ACAT inhibitors (100 nM TMP-153 or 100 nM YIC-C8-434) increased the amounts of PrP^C-Aβ complexes after 1, 2 and 4 h (Fig. 3D). Collectively, these results indicate that esterification of cholesterol is a factor in the dissociation of PrP^C-Aβ complexes.

Aβ activates synaptic $cPLA_2$

Synapse damage occurs in response to aberrant activation of $cPLA_2$ (Bate et al., 2010) and the addition of brain extract containing 1 nM $Aβ_{42}$, but not Aβ-depleted brain extract, increased the amounts of activated (phosphorylated) $cPLA_2$ in synaptosomes (Fig. 4A,B). In synaptosomes incubated with brain extracts containing 0.125–1 nM $Aβ_{42}$ there was a significant correlation between the amounts of PrP^C-Aβ complexes and activated $cPLA_2$ (Fig. 4C). The activation of $cPLA_2$ is the first step in the production of PGs including PGE_2, a bioactive lipid which causes synapse damage in cultured neurons (Bate et al., 2010). There was a significant correlation between the amounts of activated $cPLA_2$ and the concentrations of PGE_2 released by synaptosomes (Pearson's coefficient=0.89, $P<0.01$) (Fig. 4D). There were also significant correlations between the concentrations of cholesterol and the amounts of activated $cPLA_2$ (Pearson's coefficient=0.75, $P<0.01$) (Fig. 4E), and concentrations of PGE_2 (Pearson's coefficient=0.67, $P<0.01$) (Fig. 4F) in these synaptosomes.

The activation of $cPLA_2$ is associated with its migration to specific membranes by an N-terminal lipid-binding motif (Nalefski et al., 1994). Sucrose density gradients showed that in synaptosomes the addition of Aβ caused the migration of $cPLA_2$ to low density lipid rafts (Fig. 5A), without affecting the raft distribution of synaptophysin or ganglioside GM-1 (Fig. 5B). In synaptosomes, Aβ caused a dose-dependent translocation of $cPLA_2$ to rafts as defined by their resistant to cold triton X-100 (Fig. 5C). Immunoprecipitation studies showed that Aβ caused $cPLA_2$ to colocalize in PrP^C-containing rafts (Fig. 5D) (Bate and Williams, 2011). Following the addition of $Aβ_{42}$ (0.125–1 nM), there were significant correlations between the amounts of $cPLA_2$ in rafts and the amounts of activated $cPLA_2$ (Pearson's coefficient=0.92, $P<0.01$) (Fig. 5E) and cholesterol concentrations (Pearson's coefficient=0.7, $P<0.01$) (Fig. 5F). These results support the hypothesis that the Aβ-induced increase in cholesterol stabilises raft-associated signalling complexes that attract and activate $cPLA_2$.

CEH inhibitors reduce the Aβ-induced activation of $cPLA_2$

The correlations between cholesterol concentrations, raft-associated and activated $cPLA_2$ suggested a causal relationship. In synaptosomes, the Aβ-induced increase in activated $cPLA_2$ was reduced by pre-treatment with CEH inhibitors [20 µM DEUP or 5 µM cholesteryl N-(2-dimethylaminoethyl) carbamate] (Fig. 6A). By contrast, pre-treatment of synaptosomes with ACAT inhibitors (100 nM TMP-153 or 100 nM YIC-C8-434) enhanced the Aβ-induced activation of $cPLA_2$ (Fig. 6B). Similarly, pre-treatment with CEH inhibitors reduced, while ACAT inhibitors increased, the Aβ-induced release of PGE_2 from synaptosomes (Fig. 6C). Neither CEH nor ACAT inhibitors affected the activation of $cPLA_2$ by phospholipase A_2-activating peptide (PLAP, also known as PLAA), indicating they did not have a direct effect on the enzyme (Fig. 6D). The Aβ-induced translocation of $cPLA_2$ to rafts was transient (Fig. 6E) and was reduced by pre-treatment with CEH inhibitors. In synaptosomes pre-treated with ACAT inhibitors (100 nM TMP-153 or 100 nM YIC-C8-434), $cPLA_2$ remained in rafts longer than in control synaptosomes following the addition of Aβ (Fig. 6F).

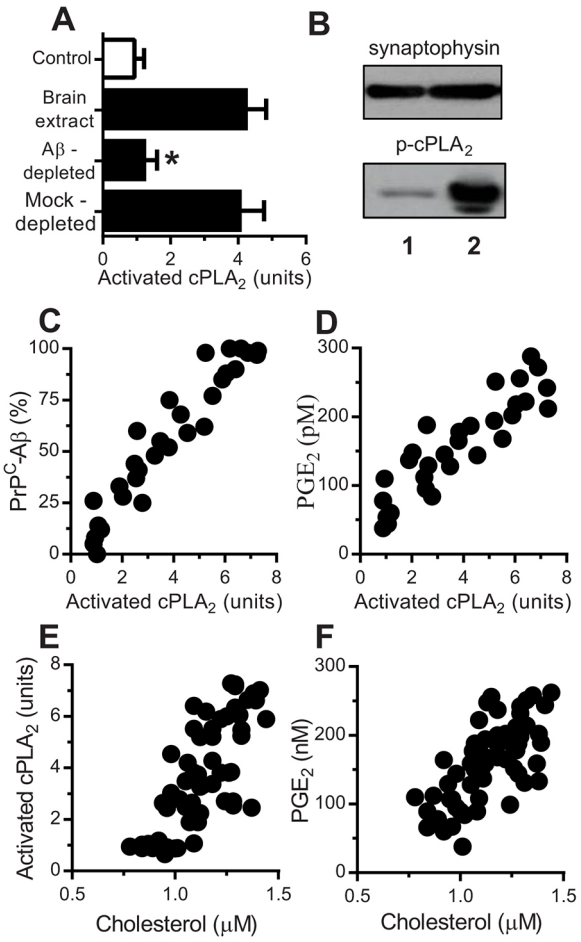

Fig. 4. Aβ-activated $cPLA_2$. (A) The amounts of activated $cPLA_2$ in synaptosomes incubated with control medium, brain extract, Aβ-depleted brain extract or mock-depleted brain extract. Values are mean±s.d. from triplicate experiments performed four times; $n=12$. The asterisk indicates significantly less activated $cPLA_2$ than in synaptosomes incubated with brain extract (*t*-test, $P<0.01$). (B) The amounts of synaptophysin and activated (phosphorylated) $cPLA_2$ in synaptosomes incubated with control medium (1) or with brain extract containing 1 nM $Aβ_{42}$ (2). In synaptosomes incubated with brain extract containing $Aβ_{42}$ (0.125–1 nM), there were significant correlations between the amounts of activated $cPLA_2$ and PrP^C-Aβ complexes (Pearson's coefficient=0.95, $P<0.01$) (C), and between the amounts of activated $cPLA_2$ and concentrations of PGE_2 (Pearson's coefficient=0.89, $P<0.01$) (D). There were also significant correlations between the concentrations of cholesterol and the amounts of activated $cPLA_2$ (Pearson's coefficient=0.75, $P<0.01$) (E), and between concentrations of cholesterol and PGE_2 (Pearson's coefficient=0.67, $P<0.01$) (F), in these synaptosomes.

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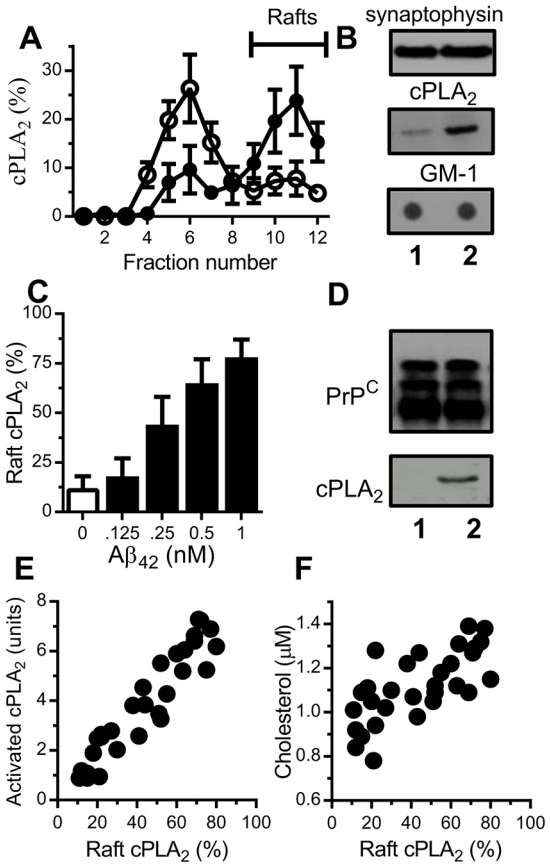

Fig. 5. Aβ causes the translocation of cPLA$_2$ to rafts. (A) The amounts of cPLA$_2$ in fractions from a sucrose density gradient derived from control synaptosomes (○) or from synaptosomes incubated with brain extract containing 1 nM Aβ$_{42}$ (●). Values are mean±s.d. from triplicate experiments performed three times; *n*=9. (B) Immunoblots showing the amounts of synaptophysin, cPLA$_2$ and GM-1 in DRMs (rafts) from synaptosomes incubated with control medium (1) or with brain extract containing 1 nM Aβ$_{42}$ (2). (C) The amounts of cPLA$_2$ in DRMs (rafts) derived from synaptosomes incubated with control medium (□) or with brain extract containing Aβ$_{42}$ as shown (■). Values are mean±s.d., from triplicate experiments performed three times; *n*=9. (D) Immunoblots showing PrP^C and cPLA$_2$ in immunoprecipitates from synaptosomes incubated with control medium (1) or with brain extract containing 1 nM Aβ$_{42}$ (2). There were significant correlations between the amounts of cPLA$_2$ in rafts and the amounts of activated cPLA$_2$ (Pearson's coefficient=0.92, *P*<0.01) (E) and the concentrations of cholesterol (Pearson's coefficient=0.7, *P*<0.01) (F) in synaptosomes incubated with brain extract containing Aβ$_{42}$ (0.125–1 nM).

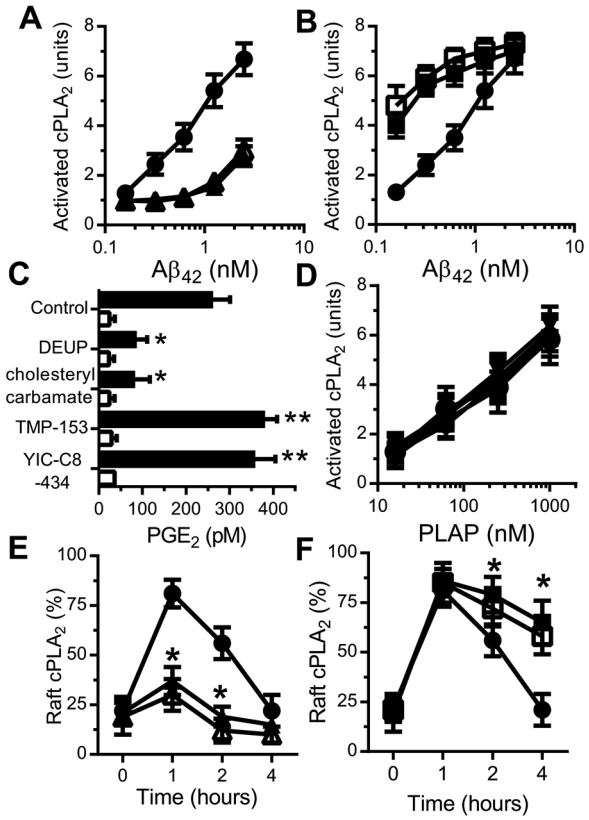

Fig. 6. CEH inhibitors reduce the Aβ-induced activation of cPLA$_2$. (A) The amounts of activated cPLA$_2$ in synaptosomes pre-treated with control medium (●), or the CEH inhibitors 20 μM DEUP (△) or 5 μM cholesteryl N-(2-dimethylaminoethyl) carbamate (▲), and incubated with brain extract containing Aβ$_{42}$ as shown. Values are mean±s.d. from triplicate experiments performed four times; *n*=12. (B) The amounts of activated cPLA$_2$ in synaptosomes pre-treated with control medium (●), or the ACAT inhibitors 100 nM TMP-153 (■) or 100 nM YIC-C8-434 (□), and incubated with brain extract containing Aβ$_{42}$ as shown. Values are mean±s.d. from triplicate experiments performed four times; *n*=12. (C) The concentrations of PGE$_2$ in synaptosomes treated with control medium, 20 μM DEUP, 5 μM cholesteryl N-(2-dimethylaminoethyl) carbamate, 100 nM TMP-153 or 100 nM YIC-C8-434, and incubated with control medium (□) or brain extract containing 1 nM Aβ$_{42}$ (■). Values are mean±s.d. from triplicate experiments performed three times; *n*=9. Concentrations of PGE$_2$ significantly less (*) or more (**) than in control synaptosomes incubated with Aβ (*t*-test, *P*<0.01). (D) The amounts of activated cPLA$_2$ in synaptosomes pre-treated with control medium (●), 20 μM DEUP (△), 5 μM cholesteryl N-(2-dimethylaminoethyl) carbamate (▲), 100 nM TMP-153 (■) or 100 nM YIC-C8-434 (□), and incubated with PLAP as shown. Values are mean±s.d. from triplicate experiments performed four times; *n*=12. (E) The amounts of cPLA$_2$ in DRMs (rafts) from synaptosomes pre-treated with control medium (●), CEH inhibitors, 20 μM DEUP (△) or 5 μM cholesteryl N-(2-dimethylaminoethyl) carbamate (▲), and incubated brain extract containing 1 nM Aβ$_{42}$ for the indicated time periods. Values are mean±s.d. from triplicate experiments performed three times; *n*=9. The asterisks indicate amounts of raft cPLA$_2$ significantly less than in control synaptosomes incubated with Aβ$_{42}$ (*t*-test, *P*<0.01). (F) The amounts of cPLA$_2$ in DRMs (rafts) from synaptosomes pre-treated with control medium (●), ACAT inhibitors, 100 nM TMP-153 (■) or 100 nM YIC-C8-434 (□), and incubated brain extract containing 1 nM Aβ$_{42}$ for the indicated time periods. Values are mean±s.d. from triplicate experiments performed three times; *n*=9. The asterisks indicate amounts of raft cPLA$_2$ significantly higher than in control synaptosomes incubated with Aβ$_{42}$ (*t*-test, *P*<0.01).

CEH inhibitors reduce Aβ-induced synapse damage

The effects of the cholesterol ester cycle on Aβ-induced synapse damage in cultured neurons were studied. Brain extracts caused the Aβ-dependent loss of synaptic proteins including synapsin-1, vesicle-associated membrane protein-1, synaptophysin and cysteine string protein (CSP, also known as DNAJC5) from cultured neurons in a tissue culture model of synapse degeneration (Osborne et al., 2016). Here, the addition of brain extracts reduced the amounts of synaptophysin (Fig. 7A) and CSP (Fig. 7B) in neurons, indicative of synapse degeneration. Pre-treatment with CEH inhibitors (20 μM DEUP or 5 μM cholesteryl N-(2-dimethylaminoethyl) carbamate) protected cultured neurons against the Aβ-induced loss of synaptophysin and CSP. By contrast, pre-treatment of neurons with 100 nM TMP-153 or 100 nM YIC-C8-434 increased the Aβ-induced loss of synaptophysin (Fig. 7C) and CSP (Fig. 7D). None of the drugs tested affected synapse damage caused by PLAP; there were no significant differences in the amounts of synaptophysin in neuronal cultures incubated with 250 nM PLAP after pre-treatment with control medium or 20 μM

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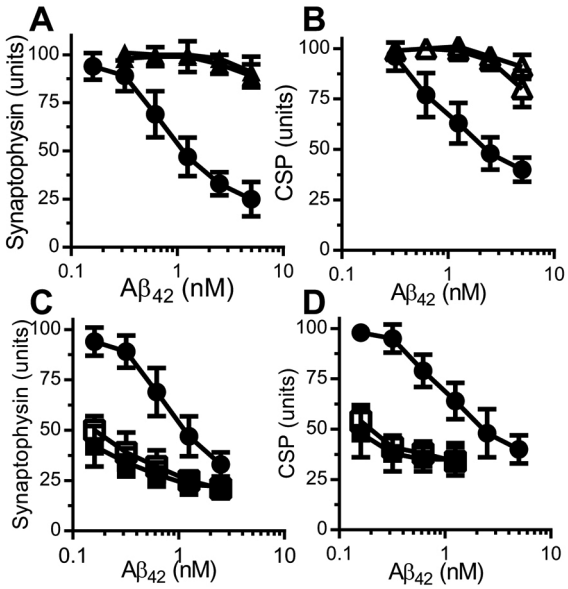

Fig. 7. CEH inhibitors protect neurons against Aβ-induced synapse damage. (A,B) The amounts of synaptophysin (A) and CSP (B) in neurons pre-treated with control medium (●), 20 µM DEUP (▲) or 5 µM cholesteryl N-(2-dimethylaminoethyl) carbamate (△), and incubated with brain extract containing $A\beta_{42}$ as shown. Values are mean±s.d. from triplicate experiments performed three times; *n*=9. (C,D,) The amounts of synaptophysin (C) and CSP (D) in neurons pre-treated with control medium (●), 100 nM TMP-153 (■) or 100 nM YIC-C8-434 (□) and incubated with brain extract containing $A\beta_{42}$ as shown. Values are mean±s.d. from triplicate experiments performed three times; *n*=9.

DEUP (31±7 units compared with 36±9 units, *P*=0.13, *n*=9) or 5 µM cholesteryl N-(2-dimethylaminoethyl) carbamate (31±7 units compared with 31±8 units, *P*=0.73, *n*=9), 100 nM TMP-153 (31±7 units compared with 36±7 units, *P*=0.16, *n*=9) or 100 nM YIC-C8-434 (31±7 units compared with 33±10 units, *P*=0.71, *n*=9).

Squalene increases Aβ-induced synapse damage

An altered cholesterol ester cycle, including the accumulation of cholesterol esters, has been reported in AD patients (Pani et al., 2009). To mimic this process, cultured neurons were fed squalene (0.6–5 µM) for 24 h and synaptosomes were isolated. The addition of 5 µM squalene did not affect concentrations of synaptophysin (98±6 units compared with 100±5 units, *n*=6, *P*=0.25) or CSP (99±4 units compared with 100±4 units, *n*=6, *P*=0.61), indicating that synaptic density was not altered. Squalene treatment increased the concentrations of cholesterol esters in synaptosomes (Fig. 8A). The addition of brain extract induced a significantly larger increase in cholesterol concentrations in synaptosomes from squalene-treated neurons than in synaptosomes from control neurons (Fig. 8B). There was a significant correlation between the cholesterol concentrations and activated $cPLA_2$ in synaptosomes derived from squalene-treated neurons incubated with brain extract containing 1 nM $A\beta_{42}$ (Pearson's coefficient=0.9, *P*<0.01) (Fig. 8C). Synaptosomes from neurons treated with squalene produced higher amounts of activated $cPLA_2$ in response to Aβ than did synaptosomes from control neurons (Fig. 8D). Finally, pre-treatment of neurons with 5 µM squalene significantly enhanced the Aβ-induced loss of synaptophysin (Fig. 8E) and CSP (Fig. 8F).

DISCUSSION

Here, we show a pivotal role for the cholesterol ester cycle in controlling the formation and dissociation of signalling complexes

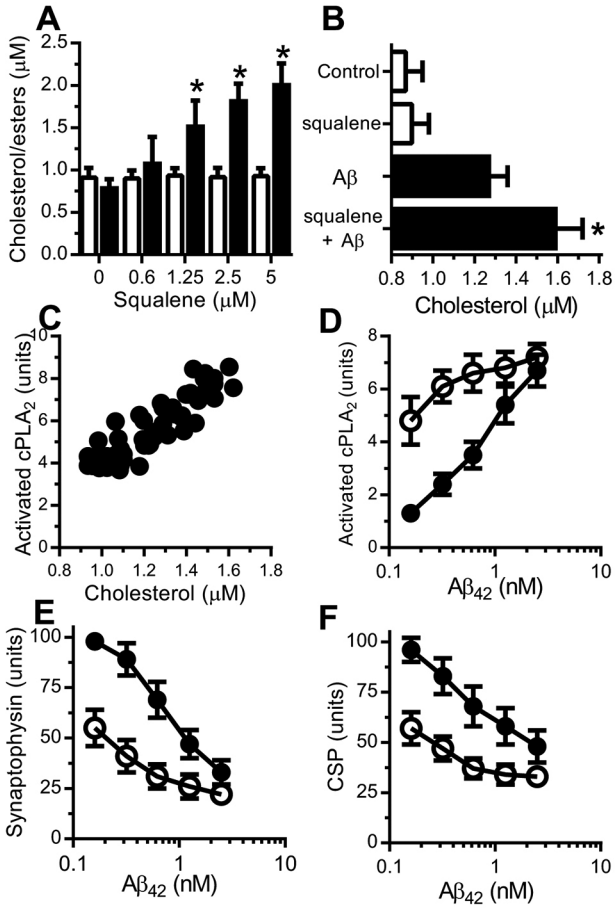

Fig. 8. Increased synaptic cholesterol esters are associated with increased Aβ-induced synapse damage. (A) The concentrations of cholesterol (□) and cholesterol esters (■) in synaptosomes derived from neurons treated with squalene. Values are mean±s.d. from triplicate experiments performed three times; *n*=9. The asterisks indicate concentrations of cholesterol esters significantly higher than in control synaptosomes (*t*-test, *P*<0.01). (B) The concentrations of cholesterol in synaptosomes derived from neurons treated with control medium or 5 µM squalene (□), and in these synaptosomes incubated with brain extract containing 1 nM $A\beta_{42}$ (■). Values are mean±s.d. from triplicate experiments performed three times *n*=9. The asterisk indicates concentrations of cholesterol significantly higher than in synaptosomes incubated with $A\beta_{42}$ (*t*-test, *P*<0.01). (C) There was a significant correlation between the concentrations of cholesterol and activated $cPLA_2$ in synaptosomes derived from neurons treated with squalene (0.6 to 5 µM) and incubated with brain extract containing 1 nM $A\beta_{42}$ (Pearson's coefficient=0.9, *P*<0.01). (D) The amounts of activated $cPLA_2$ in synaptosomes from neurons treated with control medium (●) or 5 µM squalene (○) and incubated with brain extract containing $A\beta_{42}$ as shown. Values are mean±s.d. from triplicate experiments performed three times; *n*=9. The amounts of synaptophysin (E) and CSP (F) in neurons pre-treated with control medium (●) or 5 µM squalene (○) and incubated with brain extract containing $A\beta_{42}$ as shown. Values are mean±s.d. from triplicate experiments performed three times; *n*=9.

formed in synapses in response to Aβ. More specifically, these studies demonstrate that transient changes in cholesterol concentrations control the Aβ-induced activation of $cPLA_2$ at synapses. Thus, inhibition of CEHs reduced the Aβ-induced rise in cholesterol, reduced the formation of PrP^C-Aβ complexes and the activation of $cPLA_2$ in synapses, and reduced synapse damage in neuronal cultures. Conversely, inhibiting the esterification of cholesterol accentuated the Aβ-induced increase in cholesterol, stabilised PrP^C-Aβ complexes, increased activation of $cPLA_2$ and increased synapse damage.

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It is widely believed that the concentration of cholesterol in cell membranes is a critical factor involved in neurodegeneration (Maxfield and Tabas, 2005). Here, we show that physiologically relevant concentrations of natural Aβ increased cholesterol concentrations within synaptosomes; an observation that is consistent with reports of increased cholesterol concentrations in Aβ-positive synapses in the cortex of AD patients (Gylys et al., 2007). Although the preparations used in these assays are likely to contain different fragments of the amyloid precursor protein, responses were similar when preparations were standardized according to their $A\beta_{42}$ content. The Aβ-induced increase in synaptic cholesterol was not caused by cholesterol synthesis; rather it was controlled by the cholesterol ester cycle; the Aβ-induced increase in cholesterol was accompanied by a corresponding reduction in cholesterol esters, indicating the activation of a CEH. Inhibition of the Aβ-induced increase in synaptic cholesterol by two selective CEH inhibitors supported this conclusion. Time course studies demonstrated that the Aβ-induced increase in cholesterol/reduction in cholesterol esters was transient. These experiments could not be reliably extended beyond 4 h due to the degradation of isolated synaptosomes. CEH inhibitors did not affect the concentrations of cholesterol in the absence of Aβ, indicating that the cholesterol ester hydrolysis occurs in response to specific stimuli.

The concentration of cholesterol in the cell membranes is critical for the formation of lipid raft membrane platforms that concentrate molecules involved in cell signalling (Simons and Toomre, 2000). Specific stimuli cause individual rafts to coalesce to form a larger platform capable of cell activation (Lingwood et al., 2008; Lingwood and Simons, 2010). Raft formation is associated with the oligomerization of proteins (Hammond et al., 2005), including the aggregation of PrP^C by Aβ oligomers (Bate and Williams, 2011). Notably, the increase in synaptic cholesterol concentrations was associated with the toxic Aβ oligomers (Mc Donald et al., 2010; McLean et al., 1999) rather than nontoxic Aβ monomers (Giuffrida et al., 2009). The formation of PrP^C-Aβ complexes was also transient and mirrored the Aβ-induced changes in cholesterol; there was a close temporal association between concentrations of cholesterol and PrP^C-Aβ complexes. We hypothesise that Aβ stimulates the hydrolysis of cholesterol esters resulting in the release of cholesterol that stabilises PrP^C-Aβ complexes. Thus, the inhibition of CEHs reduced the Aβ-induced rise in cholesterol and consequently the formation of PrP^C-Aβ complexes.

The activation of $cPLA_2$ is the first step in the production of PGE_2. Since the concentrations of PGE_2 are raised in the cerebrospinal fluid of patients with AD (Montine et al., 1999) and PGE_2 caused synapse damage *in vitro* (Bate et al., 2010), we propose that concentrations of Aβ >1 nM cause aberrant activation of $cPLA_2$, leading to excess PGE_2 production and synapse degeneration. This hypothesis is supported by the demonstration of significant correlations between Aβ-induced increases in cholesterol concentrations, activated $cPLA_2$ and PGE_2 in synapses. Inhibition of CEHs reduced the Aβ-induced activation of $cPLA_2$ and PGE_2 production. As neither CEH nor ACAT inhibitors affected activation of $cPLA_2$ by PLAP, we concluded that these drugs did not have a direct effect upon the enzyme. The activation of $cPLA_2$ involves its translocation to lipid rafts (Gaudreault et al., 2004), specifically rafts containing PrP^C-Aβ complexes (Bate and Williams, 2011). There were significant correlations between concentrations of cholesterol, the amounts of $cPLA_2$ in lipid rafts, the amounts of activated $cPLA_2$ and concentrations of PGE_2 following the addition of Aβ. Furthermore, time course studies demonstrated that the Aβ-induced rise in cholesterol, Aβ-PrP^C complexes and the translocation of $cPLA_2$ into rafts in synapses were transient and demonstrated a close temporal correlation. The inhibition of CEH in synaptosomes reduced both the amounts of $cPLA_2$ and the duration that $cPLA_2$ spent within rafts.

The dissociation of lipid raft platforms is a mechanism that limits cell signalling. Notably, the return of cholesterol/cholesterol ester concentrations to basal levels (indicating that cholesterol was being esterified) was closely associated with the dissociation of Aβ-PrP^C complexes and the return of $cPLA_2$ to the cytoplasm. Thus, the esterification of cholesterol limited the Aβ-induced increase of cholesterol in synapses as pharmacological inhibition of ACAT resulted in high cholesterol concentrations being maintained, increased Aβ-PrP^C complexes, increased time that $cPLA_2$ spent within rafts, increased activation of $cPLA_2$ and increased PGE_2 concentrations. Collectively, these results support the hypothesis that localised ACAT reduced synaptic cholesterol concentrations leading to the dissociation of PrP^C-Aβ complexes and hence the cessation of cell signalling. Conditions in which signalling platforms fail to dissociate may lead to sustained activation of signalling pathways, leading to cell disruption and disease. Here, we demonstrate that inhibition of ACAT increased Aβ-induced synapse damage. It is noteworthy that although this study focused upon cholesterol and $cPLA_2$, many other aspects of synaptic function are cholesterol-sensitive and may be influenced by the Aβ-induced increased cholesterol concentrations.

An altered cholesterol ester cycle in AD patients resulting in accumulation of cholesterol esters has been reported (Chan et al., 2012; Pani et al., 2009). The concentrations of cholesterol esters in synapses were increased by loading neurons with squalene. Synaptosomes from these neurons showed heightened responses to Aβ, increased concentrations of cholesterol and greater activation of $cPLA_2$. Of greater importance was the observation that Aβ caused greater synapse damage, as measured by the loss of synaptophysin and CSP, in squalene-loaded neurons when compared with control neurons. The role of ACAT in neurodegenerative diseases is complicated as it may affect different aspects of AD pathogenesis. ACAT inhibitors have been proposed as treatments for AD because they reduced the production of Aβ (Bryleva et al., 2010; Puglielli et al., 2001). However, in those studies ACAT inhibitors were used throughout the course of the experimental disease. The results presented here suggest that ACAT inhibitors might accelerate synapse damage in the presence of Aβ. Consequently, ACAT inhibitors might be able to prevent the development of AD but maybe contraindicated in the latter stages of AD, when concentrations of Aβ are already raised.

In summary, these results demonstrate the role of the cholesterol ester cycle in Aβ-induced cell signalling at synapses. The release of cholesterol from stores of cholesterol esters stabilises the complexes formed between PrP^C and Aβ that activate $cPLA_2$. Conversely, the esterification of cholesterol facilitates the dissociation of PrP^C-Aβ complexes and deactivation of $cPLA_2$.

MATERIALS AND METHODS

Primary neuronal cultures

Primary cortical neurons were prepared from the brains of mouse embryos (day 15.5) from Prnp wild-type$^{(+/+)}$ and Prnp knockout$^{(0/0)}$ mice after mechanical dissociation. Neuronal precursors were plated at 5×10^5 cells/well in 48-well plates in Hams F12 containing 5% foetal calf serum for 2 h. Cultures were shaken (600 rpm for 5 min) and non-adherent cells removed by two washes in PBS. Neurons were subsequently grown in neurobasal medium containing B27 components (Invitrogen) and nerve growth factor

(5 ng/ml) (Sigma-Aldrich) for 10 days. Immunohistochemistry revealed that ~95% of cells were neurofilament positive. Neurons were subsequently pre-treated with test compounds for either 24 h (squalene) or 1 h (CEH or ACAT inhibitors) before the addition of Aβ preparations or PLAP (Bachem). 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 an extraction buffer containing 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-Aldrich) and a phosphatase inhibitor cocktail including PP1, PP2A, microcystin LR, cantharidin and p-bromotetramisole (Sigma-Aldrich) were added, and nuclei and large fragments were removed by centrifugation (1000 ***g*** for 5 min).

Western blotting

Samples were mixed with Laemmli buffer containing β-mercaptoethanol, heated to 95°C for 5 min and proteins were separated by electrophoresis on 15% polyacrylamide gels (PAGE). Proteins were transferred onto a Hybond-P PVDF membrane by semi-dry blotting. Membranes were blocked using 10% milk powder; synaptophysin was detected with MAB368 (Millipore), PrP^C with mAb 4F2 (a gift from Professor Jaques Grassi, CEA, Saclay, France), $cPLA_2$ with goat polyclonal anti-$cPLA_2$ (Santa Cruz Biotechnology, sc-4049), phosphorylated-$cPLA_2$ with rabbit polyclonal anti-phospho-$cPLA_2$ (Cell Signaling Technology, 2831S) and GM-1 with biotinylated cholera toxin subunit B (Sigma-Aldrich, C9972) (all at 1 µg/ml). These were visualised using a 1:1000 dilution of biotinylated anti-mouse/goat/rat/rabbit IgG (Sigma-Aldrich) followed by extravidin-peroxidase and enhanced chemiluminescence.

Synaptophysin ELISA

Maxisorb immunoplates (Nunc) were coated with an anti-synaptophysin mAb (MAB368, Millipore, 1 µg/ml) and blocked with 5% milk powder. Samples were added for 1 h and bound synaptophysin was detected using rabbit polyclonal anti-synaptophysin (Abcam, ab53166, 1 µg/ml) followed by a biotinylated anti-rabbit IgG (Sigma-Aldrich), extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenol phosphate solution (Sigma-Aldrich). Absorbance was measured at 405 nm. Samples were expressed as 'units synaptophysin', where 100 units was the amount of synaptophysin in 10^6 untreated cells.

CSP ELISA

Maxisorb immunoplates were coated with 1 µg/ml of a mouse mAb to CSP (sc-136468, Santa Cruz Biotechnology) and blocked with 5% milk powder. Samples were added for 1 h and bound CSP was detected using 1 µg/ml rabbit polyclonal anti-CSP (sc-33154, Santa Cruz Biotechnology) followed by a biotinylated anti-rabbit IgG, extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenol phosphate solution. Absorbance was measured at 405 nm. Samples were expressed as 'units CSP', where 100 units was the amount of CSP in 10^6 untreated cells.

Isolation of synaptosomes

Synaptosomes were prepared on a discontinuous Percoll gradient as described (Dunkley et al., 2008). Neurons were homogenised at 4°C in 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*** at 4°C for 10 min. The supernatant was transferred to a gradient of 3, 7, 15 and 23% filtered Percoll prepared in SED solution and centrifuged at 16,000 ***g*** for 30 min at 4°C. Synaptosomes were collected from the interface of the 15% and 23% Percoll steps, and washed (16,000 ***g*** for 5 min at 4°C) and suspended in neurobasal medium containing B27 components at a concentration equivalent to 5×10^6 neurons per ml. All synaptosomes were used on the same day of preparation. After the test period, synaptosomes were homogenised in either extraction buffer (as above) or in the DRM extraction buffer (below). All synaptosomes preparations contained equal amounts of synaptophysin.

Isolation of DRMs

Detergent-resistant membranes (DRMs) were isolated by their insolubility in nonionic detergents as described (London and Brown, 2000). 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 ***g*** for 5 min at 4°C). The post-nuclear supernatant was incubated on ice (4°C) for 1 h and centrifuged (16,000 ***g*** for 30 min at 4°C). 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 and centrifuged (10 min at 16,000 ***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 ***g*** for 5 min). Membranes were washed by centrifugation at 16,000 ***g*** for 10 min at 4°C 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 50,000 ***g*** for 18 h at 4°C. Serial 1 ml aliquots were collected from the bottom of gradients.

Cholesterol measurement

The concentrations of cholesterol in samples were measured using the Amplex Red cholesterol assay kit (Life Technologies) based upon Robinet et al. (2010). Briefly, control and treated synaptosomes were washed (400 ***g***, 10 min) and lipids extracted by suspension in hexane:isopropanol (3:2, v/v) and disruption for 10 min in a cell disruptor (Disruptor Genie, Scientific Instruments). Samples were centrifuged (10,000 ***g***, 1 min), and the supernatants collected and dried under liquid nitrogen. Lipids were dissolved in 500 µl isopropanol:NP40 (9:1, v/v) and sonicated in a water bath (30 min). Samples were pre-treated with catalase before the enzyme cocktail of the Amplex Red kit [0.1 M potassium phosphate buffer pH 7.4, 0.25 M NaCl, 5 mM cholic acid, 0.1% Triton X-100, cholesterol oxidase (±cholesterol esterase), horse radish peroxidase and 0.4 mM 10-acetyl-3,7-dihydroxyphenoxazine] was added and incubated at 37°C for 30 min. Cholesterol is oxidised by cholesterol oxidase to yield hydrogen peroxide and ketones. The hydrogen peroxide reacts with 10-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red reagent) to produce highly fluorescent resorufin, which is measured by excitation at 530 nm and emission detection at 590 nm. Each experiment contained cholesterol standards and solvent only controls. Cholesterol concentrations of samples were calculated by reference to the cholesterol standards.

$cPLA_2$ ELISA/activated $cPLA_2$/PGE_2 ELISA

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 mouse mAb anti-$cPLA_2$ (clone CH-7, Upstate, 05-568) and blocked with 5% milk powder in PBS+0.1% Tween 20. Samples were incubated for 1 h and the amount of bound $cPLA_2$ was detected using 1 µg/ml goat polyclonal anti-$cPLA_2$ (Santa Cruz Biotechnology, sc-4049) followed by biotinylated anti-goat IgG, extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenol phosphate solution. Absorbance was measured at 405 nm. 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 anti-$cPLA_2$ mAb (clone CH-7, Upstate, 05-568) combined with 1 µg/ml rabbit polyclonal anti-phospho-$cPLA_2$ (Cell Signaling

Technology, 2831S), followed by biotinylated anti-rabbit IgG (Sigma-Aldrich), extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenol phosphate solution. Absorbance was measured on a microplate reader at 405 nm. Results were expressed as 'units activated $cPLA_2$' (1 unit=amount of activated $cPLA_2$ in control preparations). The amounts of PGE_2 in synaptosomes were determined using a competitive enzyme immunoassay kit (R&D Systems) according to the manufacturer's instructions.

Brain extracts

Samples of temporal lobes from three patients with a clinical and pathologically confirmed diagnosis of AD were supplied by Asterand, an international supplier of human tissue (informed consent was given to Asterand, and samples were collected according to the Declaration of Helsinki, 2000). Soluble extracts were prepared using methodology as previously described (Shankar et al., 2008). Briefly, brain tissue was cut into pieces of ∼100 mg and added to 2 ml tubes containing lysing matrix D beads (Q-Bio). Ice-cold 20 mM Tris HCl pH 7.4 containing 150 mM NaCl was added to an equivalent of 100 mg brain tissue/ml and tubes were shaken for 10 min. This process was performed three times before tubes were centrifuged at 16,000 ***g*** for 10 min to remove particulate matter. Soluble material was prepared by passage through a 50 kDa filter (Sartorius) (16,000 ***g*** for 30 min to remove proteolytic enzymes, membrane-bound and plaque Aβ). The remaining material was then desalted (3 kDa filter, Sartorius) to eliminate bioactive small molecules and drugs, and the retained material collected (preparation contains molecules with molecular weights between 3 and 50 kDa). Monomers were prepared by passage through a 10 kDa filter (Sartorius) and oligomers were collected from the material that was retained (10–50 kDa). The concentrations of Aβ in each preparation were measured by ELISA (see below). Preparations were stored at −80°C. For cell experiments, preparations were diluted in neurobasal medium containing B27 components. For immunoblots, preparations were mixed with an equal volume of 0.5% NP-40, 5 mM CHAPS, 50 mM Tris pH 7.4, and separated by PAGE. Proteins were transferred onto a Hybond-P PVDF membrane by semi-dry blotting and blocked using 10% milk powder. Aβ was detected by incubation with 1 µg/ml mAb 6E10, reactive with amino acids 1-16 of Aβ (Covance, SIG-39340), biotinylated anti-mouse IgG, extravidin-peroxidase and enhanced chemiluminescence.

Immunodepletions

Brain extracts were incubated with 1 µg/ml mAb 4G8 (reactive with amino acids 17–24 of Aβ, Covance, SIG-39220) or 1 µg/ml mAb 3F4 (reactive with human prion proteins, Millipore, MAB1562; mock-depletion) and incubated at 4°C on rollers for 24 h. Protein G microbeads were added (10 µl/ml) (Sigma-Aldrich) for 2 h, protein G bound-antibody complexes removed by centrifugation and the depleted media filtered before use.

Sample preparation for end-specific ELISAs

To detach Aβ from cellular components that occlude specific epitopes, samples (50 µl) were mixed with 250 µl 70% formic acid and sonicated. A 50 µl aliquot was added to 50 µl 10 M Tris-HCl with protease inhibitors (as above) and sonicated before addition to ELISA.

$Aβ_{40}$/$Aβ_{42}$ ELISA

Nunc Maxisorb immunoplates were coated with 1 µg/ml mAb 4G8 and blocked with 5% milk powder. Samples were added for 1 h. In separate plates, $Aβ_{40}$ was detected with 2 µg/ml rabbit polyclonal PC-149 (Merck) and $Aβ_{42}$ with a 1 µg/ml rabbit mAb BA3-9 (Covance, SIG-39168), followed by biotinylated anti-rabbit IgG, extravidin alkaline phosphatase (Sigma-Aldrich) and 1 mg/ml 4-nitrophenol phosphate solution. Absorbance was measured at 405 nm and compared to a dose response of synthetic $Aβ_{40}$/$Aβ_{42}$ (Bachem).

PrP^C:Aβ complex ELISA

Maxisorb immunoplates were coated with 1 µg/ml mAb 4F2 reactive with PrP^C (a gift from Professor Jaques Grassi). Plates were blocked with 5% milk powder and samples were added for 1 h. Aβ bound to PrP^C was detected by 1 µg/ml biotinylated mAb 6E10 (reactive with epitopes 1–16 of Aβ, Covance), followed by extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenol phosphate solution. Absorbance was measured at 405 nm. Samples were expressed as a % of the maximum OD in control synaptosomes.

Drugs

Stock solutions were dissolved in ethanol or DMSO, and diluted in culture medium to obtain final working concentrations. Vehicle controls consisted of equivalent dilutions of ethanol or DMSO.

Statistical analysis

Comparison of treatment effects was carried out using Student's paired *t*-tests, and one-way and two-way ANOVA with Bonferroni's post hoc tests (IBM SPSS Statistics 20). Data are presented as mean±s.d. and $P<0.01$ was considered significant. Correlations between data sets were analysed using Pearson's bivariate coefficient (IBM SPSS Statistics 20).

Competing interests

The authors declare no competing or financial interests.

Author contributions

Methodology: E.W., C.O., C.B.; Formal analysis: E.W., C.O., C.B.; Investigation: E.W., C.O., C.B.; Writing - original draft: C.B.; Writing - review & editing: C.B.; Supervision: C.B.

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References

Anderson, R. G. W. and Jacobson, K. (2002). A role for lipid shells in targeting proteins to caveolae, rafts, and other lipid domains. *Science* **296**, 1821-1825.
Bate, C. and Williams, A. (2011). Amyloid-β-induced synapse damage is mediated via cross-linkage of cellular prion proteins. *J. Biol. Chem.* **286**, 37955-37963.
Bate, C. and Williams, A. (2012). Neurodegeneration induced by clustering of sialylated glycosylphosphatidylinositols of prion proteins. *J. Biol. Chem.* **287**, 7935-7944.
Bate, C., Tayebi, M. and Williams, A. (2010). Phospholipase A_2 inhibitors protect against prion and Aβ mediated synapse degeneration. *Mol. Neurodegener.* **5**, 13.
Baxter, A., Fitzgerald, B. J., Hutson, J. L., McCarthy, A. D., Motteram, J. M., Ross, B. C., Sapra, M., Snowden, M. A., Watson, N. S. and Williams, R. J. (1992). Squalestatin 1, a potent inhibitor of squalene synthase, which lowers serum cholesterol in vivo. *J. Biol. Chem.* **267**, 11705-11708.
Bryleva, E. Y., Rogers, M. A., Chang, C. C. Y., Buen, F., Harris, B. T., Rousselet, E., Seidah, N. G., Oddo, S., LaFerla, F. M., Spencer, T. A. et al. (2010). ACAT1 gene ablation increases 24(S)-hydroxycholesterol content in the brain and ameliorates amyloid pathology in mice with AD. *Proc. Natl. Acad. Sci. USA* **107**, 3081-3086.
Chan, R. B., Oliveira, T. G., Cortes, E. P., Honig, L. S., Duff, K. E., Small, S. A., Wenk, M. R., Shui, G. and Di Paolo, G. (2012). Comparative lipidomic analysis of mouse and human brain with Alzheimer disease. *J. Biol. Chem.* **287**, 2678-2688.
Chang, T.-Y., Chang, C. C. Y., Ohgami, N. and Yamauchi, Y. (2006). Cholesterol sensing, trafficking, and esterification. *Annu. Rev. Cell Dev. Biol.* **22**, 129-157.
Chen, C. and Bazan, N. G. (2005). Endogenous PGE_2 regulates membrane excitability and synaptic transmission in hippocampal CA1 pyramidal neurons. *J Neurphysiol* **93**, 929-941.
Chen, Y., Thelin, W. R., Yang, B., Milgram, S. L. and Jacobson, K. (2006). Transient anchorage of cross-linked glycosyl-phosphatidylinositol-anchored proteins depends on cholesterol, Src family kinases, caveolin, and phosphoinositides. *J. Cell Biol.* **175**, 169-178.
Chiarini, L. B., Freitas, A. R., Zanata, S. M., Brentani, R. R., Martins, V. R. and Linden, R. (2002). Cellular prion protein transduces neuroprotective signals. *EMBO J.* **21**, 3317-3326.
Chiesa, R., Piccardo, P., Biasini, E., Ghetti, B. and Harris, D. A. (2008). Aggregated, wild-type prion protein causes neurological dysfunction and synaptic abnormalities. *J. Neurosci.* **28**, 13258-13267.
Dunkley, P. R., Jarvie, P. E. and Robinson, P. J. (2008). A rapid Percoll gradient procedure for preparation of synaptosomes. *Nat. Protoc.* **3**, 1718-1728.
Gaudreault, S. B., Chabot, C., Gratton, J.-P. and Poirier, J. (2004). The caveolin scaffolding domain modifies 2-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor binding properties by inhibiting phospholipase A_2 activity. *J. Biol. Chem.* **279**, 356-362.
Gilch, S., Kehler, C. and Schätzl, H. M. (2006). The prion protein requires cholesterol for cell surface localization. *Mol. Cell. Neurosci.* **31**, 346-353.

Giuffrida, M. L., Caraci, F., Pignataro, B., Cataldo, S., De Bona, P., Bruno, V., Molinaro, G., Pappalardo, G., Messina, A., Palmigiano, A. et al. (2009). β–amyloid monomers are neuroprotective. *J. Neurosci.* **29**, 10582-10587.

Gocze, P. M. and Freeman, D. A. (1992). A cholesteryl ester hydrolase inhibitor blocks cholesterol translocation into the mitochondria of MA-10 Leydig tumor cells. *Endocrinology* **131**, 2972-2978.

Gylys, K. H., Fein, J. A., Yang, F., Miller, C. A. and Cole, G. M. (2007). Increased cholesterol in A[beta]-positive nerve terminals from Alzheimer's disease cortex. *Neurobiol. Aging* **28**, 8-17.

Hammond, A. T., Heberle, F. A., Baumgart, T., Holowka, D., Baird, B. and Feigenson, G. W. (2005). Crosslinking a lipid raft component triggers liquid ordered-liquid disordered phase separation in model plasma membranes. *Proc. Natl. Acad. Sci. USA* **102**, 6320-6325.

Herms, J., Tings, T., Gall, S., Madlung, A., Giese, A., Siebert, H., Schurmann, P., Windl, O., Brose, N. and Kretzschmar, H. (1999). Evidence of presynaptic location and function of the prion protein. *J. Neurosci.* **19**, 8866-8875.

Hosie, L., Sutton, L. D. and Quinn, D. M. (1987). p-Nitrophenyl and cholesteryl-N-alkyl carbamates as inhibitors of cholesterol esterase. *J. Biol. Chem.* **262**, 260-264.

Kaneko, K., Uchida, K., Kobayashi, T., Miura, K., Tanokura, K., Hoshino, K., Kato, I., Onoue, M. and Yokokura, T. (2001). Sex-dependent toxicity of a novel Acyl-CoA:cholesterol acyltransferase inhibitor, YIC-C8-434, in relation to sex-specific forms of cytochrome P450 in rats. *Toxicol. Sci.* **64**, 259-268.

Kawarabayashi, T., Shoji, M., Younkin, L. H., Wen-Lang, L., Dickson, D. W., Murakami, T., Matsubara, E., Abe, K., Ashe, K. H. and Younkin, S. G. (2004). Dimeric amyloid β protein rapidly accumulates in lipid rafts followed by apolipoprotein E and phosphorylated Tau accumulation in the Tg2576 mouse model of Alzheimer's disease. *J. Neurosci.* **24**, 3801-3809.

Koch, H., Huh, S.-E., Elsen, F. P., Carroll, M. S., Hodge, R. D., Bedogni, F., Turner, M. S., Hevner, R. F. and Ramirez, J.-M. (2010). Prostaglandin E2-induced synaptic plasticity in neocortical networks of organotypic slice cultures. *J. Neurosci.* **30**, 11678-11687.

Laurén, J., Gimbel, D. A., Nygaard, H. B., Gilbert, J. W. and Strittmatter, S. M. (2009). Cellular prion protein mediates impairment of synaptic plasticity by amyloid-β oligomers. *Nature* **457**, 1128-1132.

Linden, R., Cordeiro, Y. and Lima, L. M. T. R. (2012). Allosteric function and dysfunction of the prion protein. *Cell. Mol. Life Sci.* **69**, 1105-1124.

Linetti, A., Fratangeli, A., Taverna, E., Valnegri, P., Francolini, M., Cappello, V., Matteoli, M., Passafaro, M. and Rosa, P. (2010). Cholesterol reduction impairs exocytosis of synaptic vesicles. *J. Cell Sci.* **123**, 595-605.

Lingwood, D. and Simons, K. (2010). Lipid rafts as a membrane-organizing principle. *Science* **327**, 46-50.

Lingwood, D., Ries, J., Schwille, P. and Simons, K. (2008). Plasma membranes are poised for activation of raft phase coalescence at physiological temperature. *Proc. Natl. Acad. Sci. USA* **105**, 10005-10010.

London, E. and Brown, D. A. (2000). Insolubility of lipids in Triton X-100: physical origin and relationship to sphingolipid/cholesterol membrane domains (rafts). *Biochim. Biophys. Acta* **1508**, 182-195.

Maglio, L. E., Martins, V. R., Izquierdo, I. and Ramirez, O. A. (2006). Role of cellular prion protein on LTP expression in aged mice. *Brain Res.* **1097**, 11-18.

Maxfield, F. R. and Tabas, I. (2005). Role of cholesterol and lipid organization in disease. *Nature* **438**, 612-621.

Mc Donald, J. M., Savva, G. M., Brayne, C., Welzel, A. T., Forster, G., Shankar, G. M., Selkoe, D. J., Ince, P. G. and Walsh, D. M. (2010). The presence of sodium dodecyl sulphate-stable Aβ dimers is strongly associated with Alzheimer-type dementia. *Brain* **133**, 1328-1341.

McLean, C. A., Cherny, R. A., Fraser, F. W., Fuller, S. J., Smith, M. J., Vbeyreuther, K., Bush, A. I. and Masters, C. L. (1999). Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. *Ann. Neurol.* **46**, 860-866.

Montine, T. J., Sidell, K. R., Crews, B. C., Markesbery, W. R., Marnett, L. J., Roberts, L. J. and Morrow, J. D. (1999). Elevated CSF prostaglandin E2 levels in patients with probable AD. *Neurology* **53**, 1495-1498.

Moskowitz, N., Puszkin, S. and Schook, W. (1983). Characterization of brain synaptic vesicle phospholipase A_2 activity and its modulation by calmodulin, prostaglandin E_2, prostaglandin F_2 alpha, cyclic AMP, and ATP. *J. Neurochem.* **41**, 1576-1586.

Mouillet-Richard, S., Ermonval, M., Chebassier, C., Laplanche, J. L., Lehmann, S., Launay, J. M. and Kellermann, O. (2000). Signal transduction through prion protein. *Science* **289**, 1925-1928.

Nalefski, E. A., Sultzman, L. A., Martin, D. M., Kriz, R. W., Towler, P. S., Knopf, J. L. and Clark, J. D. (1994). Delineation of two functionally distinct domains of cytosolic phospholipase A2, a regulatory Ca(2+)-dependent lipid-binding domain and a Ca(2+)-independent catalytic domain. *J. Biol. Chem.* **269**, 18239-18249.

Naslavsky, N., Stein, R., Yanai, A., Friedlander, G. and Taraboulos, A. (1997). Characterization of detergent-insoluble complexes containing the cellular prion protein and its scrapie isoform. *J. Biol. Chem.* **272**, 6324-6331.

Osborne, C., West, E., Nolan, W., McHale-Owen, H., Williams, A. and Bate, C. (2016). Glimepiride protects neurons against amyloid-β-induced synapse damage. *Neuropharmacology* **101**, 225-236.

Pani, A., Dessì, S., Diaz, G., La Colla, P., Abete, C., Mulas, C., Angius, F., Cannas, M. D., Orru, C. D., Cocco, P. L. et al. (2009). Altered cholesterol ester cycle in skin fibroblasts from patients with Alzheimer's disease. *J. Alzheimers Dis.* **18**, 829-841.

Puglielli, L., Konopka, G., Pack-Chung, E., Ingano, L. A. M. K., Berezovska, O., Hyman, B. T., Chang, T. Y., Tanzi, R. E. and Kovacs, D. M. (2001). Acyl-coenzyme A: cholesterol acyltransferase modulates the generation of the amyloid beta-peptide. *Nat. Cell Biol.* **3**, 905-912.

Rajendran, L. and Simons, K. (2005). Lipid rafts and membrane dynamics. *J. Cell Sci.* **118**, 1099-1102.

Robinet, P., Wang, Z., Hazen, S. L. and Smith, J. D. (2010). A simple and sensitive enzymatic method for cholesterol quantification in macrophages and foam cells. *J. Lipid Res.* **51**, 3364-3369.

Ryan, S. D., Whitehead, S. N., Swayne, L. A., Moffat, T. C., Hou, W., Ethier, M., Bourgeois, A. J. G., Rashidian, J., Blanchard, A. P., Fraser, P. E. et al. (2009). Amyloid-β42 signals tau hyperphosphorylation and compromises neuronal viability by disrupting alkylacylglycerophosphocholine metabolism. *Proc. Natl. Acad. Sci. USA* **106**, 20936-20941.

Schroeder, R., London, E. and Brown, D. (1994). Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. *Proc. Natl. Acad. Sci. USA* **91**, 12130-12134.

Selkoe, D. J. (2002). Alzheimer's disease is a synaptic failure. *Science* **298**, 789-791.

Shankar, G. M., Li, S., Mehta, T. H., Garcia-Munoz, A., Shepardson, N. E., Smith, I., Brett, F. M., Farrell, M. A., Rowan, M. J., Lemere, C. A. et al. (2008). Amyloid-β protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nat. Med.* **14**, 837-842.

Sharma, P., Varma, R., Sarasij, R. C., Ira, Gousset, K., Krishnamoorthy, G., Rao, M. and Mayor, S. (2004). Nanoscale organization of multiple GPI-anchored proteins in living cell membranes. *Cell* **116**, 577-589.

Simons, K. and Toomre, D. (2000). Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* **1**, 31-39.

Solforosi, L., Criado, J. R., McGavern, D. B., Wirz, S., Sanchez-Alavez, M., Sugama, S., DeGiorgio, L. A., Volpe, B. T., Wiseman, E., Abalos, G. et al. (2004). Cross-linking cellular prion protein triggers neuronal apoptosis in vivo. *Science* **303**, 1514-1516.

Stahl, N., Borchelt, D. R., Hsiao, K. and Prusiner, S. B. (1987). Scrapie prion protein contains a phosphatidylinositol glycolipid. *Cell* **51**, 229-240.

Sugiyama, Y., Ishikawa, E., Odaka, H., Miki, N., Tawada, H. and Ikeda, H. (1995). TMP-153, a novel ACAT inhibitor, inhibits cholesterol absorption and lowers plasma cholesterol in rats and hamsters. *Atherosclerosis* **113**, 71-78.

Suzuki, K. G. N., Fujiwara, T. K., Sanematsu, F., Iino, R., Edidin, M. and Kusumi, A. (2007). GPI-anchored receptor clusters transiently recruit Lyn and Gα for temporary cluster immobilization and Lyn activation: single-molecule tracking study 1. *J. Cell. Biol* **177**, 717-730.

Taraboulos, A., Scott, M., Semenov, A., Avrahami, D., Laszlo, L., Prusiner, S. B. and Avraham, D. (1995). Cholesterol depletion and modification of COOH-terminal targeting sequence of the prion protein inhibit formation of the scrapie isoform. *J. Cell Biol.* **129**, 121-132.

Walsh, D. M., Klyubin, I., Fadeeva, J. V., Cullen, W. K., Anwyl, R., Wolfe, M. S., Rowan, M. J. and Selkoe, D. J. (2002). Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* **416**, 535-539.

Williamson, R., Usardi, A., Hanger, D. P. and Anderton, B. H. (2008). Membrane-bound β-amyloid oligomers are recruited into lipid rafts by a fyn-dependent mechanism. *FASEB J.* **22**, 1552-1559.

Yang, T., Li, S., Xu, H., Walsh, D. M. and Selkoe, D. J. (2017). Large soluble oligomers of amyloid β-protein from Alzheimer brain are far less neuroactive than the smaller oligomers to which they dissociate. *J. Neurosci.* **37**, 152-163.