Amyloid precursor protein controls cholesterol turnover needed for neuronal activity

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Perturbation of lipid metabolism favours progression of Alzheimer disease, in which processing of Amyloid Precursor Protein (APP) has important implications. APP cleavage is tightly regulated by cholesterol and APP fragments regulate lipid homeostasis. Here, we investigated whether up or down regulation of full-length APP expression affected neuronal lipid metabolism. Expression of APP decreased HMG-CoA reductase (HMGCR)-mediated cholesterol biosynthesis and SREBP mRNA levels, while its down regulation had opposite effects. APP and SREBP1 co-immunoprecipitated and co-localized in the Golgi. This interaction prevented Site-2 protease-mediated processing of SREBP1, leading to inhibition of transcription of its target genes. A GXXXG motif in APP sequence was critical for regulation of HMGCR expression. In astrocytes, APP and SREBP1 did not interact nor did APP affect cholesterol biosynthesis. Neuronal expression of APP decreased both HMGCR and cholesterol 24-hydroxylase mRNA levels and consequently cholesterol turnover, leading to inhibition of neuronal activity, which was rescued by geranylgeraniol, generated in the mevalonate pathway, in both APP expressing and mevastatin treated neurons. We conclude that APP controls cholesterol turnover needed for neuronal activity.

INTRODUCTION

Processing of amyloid precursor protein (APP) by β- and γ-secretase activities produces β-amyloid (Aβ) peptide, which accumulates in extracellular senile plaques present in brain of patients with Alzheimer disease (AD). Although APP processing by secretase activities has been extensively studied (Esler & Wolfe, 2001), the function of the protein remains unclear. APP knockout mice have a normal phenotype with only subtle defects, resulting from functional compensation by APP-like proteins 1 and 2 (Heber et al, 2000). The γ-secretase-mediated cleavage of APP releases the APP intracellular domain (AICD), which controls the transcription of several genes [for review see (Muller et al, 2008)] by mechanisms that could involve epigenetic modifications (Huysseune et al, 2009).

Recently, genome wide association studies (GWAS) on AD confirmed that ApoE4 is a major risk factor and provided evidence for other risk genes including Clusterin (CLU) and ABCA7 (Harold et al, 2009; Hollingworth et al, 2011; Lambert et al, 2009). The main lipoproteins in brain are ApoE and clusterin, and ABCA7 is involved in lipids efflux from cells to lipoproteins. The identification of these susceptibility loci

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As shown in Fig 1A, adenoviral expression of APP in primary cultures of rat cortical neurons increased by 60% the total APP content (1.6 ± 0.3, n = 5). This resulted in a major inhibition in cholesterol synthesis, measured by incorporation of [14C] acetate (Fig 1B), readily explained by a strong reduction in HMGCR mRNA levels (Fig 1C). A significant decrease in fatty acids synthesis was also measured (Supporting Information Fig 1). The decrease in HMGCR mRNA levels was specific, not observed when neurons were infected by a control adenovirus encoding β-galactosidase (Fig 1C), occurred as soon as APP was expressed, and did not result from transient cholesterol overload, as measured by unchanged cholesterol content over time (Supporting Information Fig 2). Expression of APP carrying the Swedish mutation (APP Swe, Fig 1D), which produces more extracellular Aβ (Johnston et al, 1994), decreased HMG-CoA reductase (HMGCR) mRNA levels to similar extent as APP (Fig 1E).

Since the intracellular domain of APP (AICD) is a transcriptional regulator, we next studied whether it could account for APP-dependent repression of HMGCR gene transcription. Neuronal expression of APP mutants in which AICD was deleted (APPΔC; Fig 1D) decreased HMGCR mRNA levels to similar extent as full-length APP (Fig 1E).

Furthermore, inhibition of AICD release by the γ-secretase inhibitor, DAPT (Dovey et al, 2001), which induced accumulation of APP C-terminal fragments in neurons expressing full-length APP (Fig 1F) and significantly decreased both rodent and human Aβ secretion (Fig 1H), did not affect APP-mediated down regulation of HMGCR mRNA levels (Fig 1G). These two lines of evidence ruled out that APP-mediated decrease in HMGCR mRNA levels is γ-secretase or AICD dependent.

Membrane-bound transcription factors known as SREBP controls HMGCR gene transcription. We therefore studied whether APP expression could affect expression of different SREBP members. SREBP1a and 2 are both actors in the regulation of cholesterol synthesis. Their mRNA levels were both decreased upon neuronal expression of APP (Fig 1I). SREBP1c, which primarily controls lipogenesis, was also specifically down-regulated upon neuronal expression of APP (Fig 1I). Despite testing several anti-SREBP2 antibodies, we were unable to detect SREBP2 protein in neurons in primary cultures. However, there is important overlap between SREBP1 and SREBP2-mediated control of transcription, and high neuronal expression of SREBP1 has been demonstrated in rodent and primate brain (Ong et al, 2000). Anti-SREBP1 antibodies do not discriminate between SREBP1a and 1c produced by alternative splicing of the same gene, but revealed changes in SREBP1 protein in cellular extracts. When APP was expressed in primary cultures of rat cortical neurons (Fig 1J), a 70% significant decrease in SREBP1 was measured in cellular extracts (Fig 1K). To further investigate the ability of APP to control expression of SREBP in neurons, primary cultures of neurons were infected with lentiviruses encoding APP specific shRNA. Acute down regulation of endogenous APP expression did not modify expression levels of APLP1 and APLP2 (Fig 1L), but induced a significant increase in HMGCR mRNA levels (Fig 1M), cholesterol biosynthesis (Fig 1N) and SREBP1 expression (Fig 1O). Up regulation of SREBP1 expression in...
neurons from APP knockout (APP<sup>−/−</sup>) mice was much less important than that observed following down regulation of endogenous APP by shRNA. (Supporting Information Fig 3A and B). In APP knockout neurons, a very significant up regulation of APLP1 (150±6%, n=3) but not APLP2 was observed (Supporting Information Fig 3C), suggesting partial compensation of absent APP by APLP1. Rescue of APP by adenoviral expression of APP in APP<sup>−/−</sup>/C0 neurons decreased SREBP1 by 80% (Supporting Information Fig 3A and B).

From these results, we conclude that APP controls neuronal cholesterol synthesis, via the SREBP pathway, by a γ-secretase- and AICD-independent mechanism.

Interaction between neuronal APP and SREBP1 in the Golgi prevents S2P-mediated release of mature SREBP1
Following translocation of membrane-bound SREBP1 from the ER to the Golgi, SREBP1 is cleaved in two steps to release its soluble mature active form, mSREBP1. Nuclear translocation of mSREBP1 then activates gene transcription. Target genes include the HMGR/S, LDLR and SREBF1 gene itself. Since adenoviral expression of APP in neurons also repressed transcription of genes encoding LDLR and HMGCs (see Supporting information Fig 4A and B), we hypothesized that increased expression of APP in neurons would inhibit proteolytic release of mSREBP1 and/or its nuclear translocation.
Following subcellular fractionation experiments, a 73 ± 4% decrease in nuclear mSREBP1 was measured in APP expressing neurons (Fig 2A). On the contrary, shRNA-mediated down regulation of endogenous APP expression induced a 127 ± 16% increase in nuclear mSREBP1 (Fig 2B).

Immunofluorescence using the H160 antibody raised against the N-terminus of SREBP1, which recognizes mSREBP1, clearly showed a nuclear localization of mSREBP1 in control neurons (Fig 2C and E). In contrast, a more cytoplasmic distribution of SREBP1 was observed in APP-expressing neurons (Fig 2F and H). These results confirmed that expression of APP in neurons not only decreased SREBP1 expression but also prevented nuclear mSREBP1 localization.

Processing into mSREBP1 requires SREBP1 translocation from the ER to the Golgi. To test whether inhibition of mSREBP1 production by APP could result from its retention together with APP in the ER, the neuronal localization of SREBP1 and APP was analysed by immunofluorescence. In APP-expressing neurons, the N-terminus of SREBP1 co-localized with APP, as well as with TGN46, a Golgi marker (Fig 2J–K). An antibody raised against the C-terminus of SREBP1 (C20) showed similar co-localization (Supporting Information Fig 5A–C), indicating that full length SREBP1 co-localized with APP in the Golgi. These results demonstrate that APP expression does not prevent SREBP1 translocation from the ER to the Golgi, but presumably causes its retention as full-length protein in the Golgi. We therefore tested for an interaction between these two proteins. APP and APP C99 were found to co-immunoprecipitate with SREBP1 (Fig 2L–N). mSREBP1 was associated with APP and APP C99 in neurons (Fig 2M). In addition, APP also co-immunoprecipitated with SCAP (Fig 2O). Moreover, SREBP1 co-immunoprecipitated with APP (Fig 2P), whereas the ER chaperone protein GRP78 was not detected with SCAP (Fig 2Q). These results indicate that SREBP1 interacts with APP and APP C99 in neurons. This interaction does not result from overexpression of APP, since the same interaction between endogenous neuronal APP and SCAP, and SREBP1 was demonstrated in co-immunoprecipitation experiments (Supporting Information Fig 6).

Altogether, these results indicated that, in neurons, interaction between APP and SREBP1 in the Golgi prevents Site-2 protease-mediated release and nuclear translocation of mature SREBP1.

A GXXXG motif of the transmembrane domain of APP is critical for SREBP1-mediated regulation of HMGCR expression

In order to define the domain of APP interacting with SREBP1, rat cortical neurons were infected with recombinant adenoviruses encoding full-length APP or a mutant APP in which the C- or N-terminal domain was deleted (APPΔC or C99, respectively). In co-immunoprecipitation experiments, APP, APPΔC, and C99 were all found to interact with SREBP1 (Fig 3A). As the sequence common to these constructs is the juxta/transmembrane domain of APP, this APP sequence seems important for APP:SREBP1 interaction, which prevents SREBP1 processing by S2P.

A recent study identified critical amino acids in the N-terminal portion of the C99 for direct binding to cholesterol (Barrett et al, 2012). In this study, structural analyses demonstrated that G700 and G704 (APP770 numbering) located in the tandem GXXXG motifs of the transmembrane domain of C99 are essential to cholesterol binding. To investigate the importance of this GXXXG motif in the APP-mediated regulation of HMGCR expression, C99 and G625L/G629L C99 mutant (APP 695 numbering; Kienlen-Campard et al, 2008; Fig 3B) were expressed in rat cortical neurons at very similar levels (Fig 3C). Contrary to the C99 mutant, neuronal expression of C99 down regulated SREBP1 levels to 63 ± 8% (n = 4; Fig 3C and D) and HMGCR mRNA levels to 45 ± 6% (n = 3; Fig 3E). Interestingly, C99, but not C99 mutant, co-immunoprecipitated with SREBP1 (Fig 3F). These results indicate that a GXXXG motif of C99, predicted from structural analysis to be essential to cholesterol binding, is critical for SREBP1-mediated regulation of HMGCR expression.

APP does not affect S2P-mediated processing of ATF6

To investigate whether expression of APP would modify processing of other substrates of S2P, we measured transcription of ATF6 target genes. During ER stress, ATF6, another substrate of S2P, migrates from the ER to the Golgi (Haze et al, 1999), to be cleaved into its transcriptionally active mature form, mATF6, which also translocates into the nucleus and activates transcription of genes encoding ER-localized molecular chaperones like BIP/grp78 (Kohn et al, 1993) or the sarcoplasmic/ER Ca2+/ATPase 2 (SERCA2; Thuerauf et al, 2001). BIP/grp78 and SERCA2 mRNA levels were not affected by neuronal expression of APP.
Figure 2.
of APP (Fig 4A). In co-immunoprecipitation experiments, APP and ATF6 did not interact, whereas APP was able to interact with SREBP1 (Fig 4B) and glycosylated and non-glycosylated forms of ATF6 were present in membrane fractions of control and APP expressing neurons (Fig 4C), even if reduction of ATF6 was observed in APP expressing neurons. We can therefore conclude that APP expression does not induce ER stress, and does not affect S2P-mediated processing of ATF6.

APP does not affect SREBP1-mediated cholesterol synthesis in astrocytes

Since astrocytes are a major lipid source in the brain, we asked whether APP could also control cholesterol biosynthesis in astrocytes. In marked contrast with neurons, although adeno-viral expression of APP in primary cultures of rat cortical astrocytes (Fig 5A) increased the total APP content to very similar extent as in neurons (1.5 ± 0.2, n = 3), it did not affect cholesterol biosynthesis, by 14C acetate incorporation, nor HMGCR mRNA levels (Fig 5B and C).

Expression of APP in astrocytes (Fig 5D), or down regulation of endogenous APP expression (Fig 5E), did not modify total SREBP1 content (Fig 5D and E). Similarly, expression of APP did not modify mSREBP1 in the nuclear fraction (Fig 5F), nor induce a redistribution of SREBP1, nor inhibited nuclear mSREBP1 production (Fig 5J–L). In addition, no co-localization of APP and SREBP1 could be observed (Fig 5M–Q), and these proteins did
not co-immunoprecipitate (Fig 5R and S). We conclude that APP does not affect cholesterol synthesis in astrocytes, because SREBP1 and APP do not interact in these cells. This experiment further indicated that SREBP1:APP interaction observed in neurons was specific and occurred in living cells rather than after lysis.

**APP controls in vivo expression of SREBP1 in mice and man**

In brain samples of 5XFAD transgenic mice, a twofold decrease in SREBP1 was found in Western blotting as compared to wild type mice from the same genetic background (Fig 6A). These mice express human APP with three different mutations (Swedish, Florida and London) as well as human PS1 with 2 different mutations (PSEN1*1M146L*L286V) and develop a severe amyloid pathology at 3 months (Oakley et al, 2006). A similar decrease in SREBP1 was observed in another transgenic mouse model expressing human APP carrying the London mutation as the single transgene and developing amyloid pathology at a later age (Moechars et al, 1999; Fig 6B).

In human brain samples, SREBP1 precursor was detected as a 125 kDa protein, with different levels of expression (Fig 6C). When APP and SREBP1 signals were quantified, an inverse correlation was observed (Fig 6C). A similar inverse correlation was also observed in brain sample from an AD case with microduplication of the APP locus, in which increase in APP was concomitant with decrease in SREBP1 expression (Fig 6D and E). These results indicate that APP inversely correlates with SREBP1 in mice and man.

**APP affects neuronal cholesterol turnover and neuronal activity**

Since expression of APP inhibits cholesterol biosynthesis specifically in neurons, we further analysed the mevalonate pathway in these cells (Fig 7A). Contrary to other cell types in the brain, neurons specifically express the CYP46A1 gene encoding the cholesterol 24-hydroxylase (Lund et al, 1999), which transforms cholesterol into 24S-hydroxycholesterol. Consequently, the turnover of cholesterol is very important in neurons, resulting from equilibrium between biosynthesis and hydroxylation. Although expression of APP in neurons decreased by 90% the biosynthesis of cholesterol (Fig 7B), the content of membrane cholesterol was not affected (Fig 7B). We therefore looked at a possible down regulation of cholesterol 24-hydroxylase expression by APP. Expression of APP (Fig 7D) decreased CYP46A1 mRNA level (Fig 7F) to a very similar extent as that of HMGCGR (Fig 7E). On the contrary, two different shRNA induced acute down regulation of endogenous APP expression at different levels (Fig 7G), with a concomitant and proportional up regulation of HMGCGR and CYP46A1 mRNA levels (Fig 7H). In addition, genetic ablation of APP did not modify neuronal membrane cholesterol content (Fig 7C). Consequently, APP does not modify membrane cholesterol content but controls neuronal cholesterol turnover.

APP proteolytic processing not only regulates lipid metabolism (Grimm et al, 2012) but also synaptic transmission and ion channel function (Dawson et al, 1999; Wang et al, 2005). We previously reported that APP controls neuronal activity measured by spontaneous and synchronous calcium oscillations observed in mature neuronal networks (Santos et al, 2009). In this study, we observed that the resting potential of APP expressing neurons was not affected, while a significant increase in L-type calcium currents was measured in these neurons. Resulting calcium influx activated calcium-dependent potassium channels (SK channels) and increased the p.d. amplitude of medium after hyper polarization leading to inhibition of calcium oscillations in APP expressing neuronal networks (Santos et al, 2009). Accordingly, expression of APP in rat cortical neuronal networks inhibited calcium oscillations, while shRNA-mediated acute down regulation of endogenous APP increased the frequency of oscillations and decreased their amplitude (Fig 7I–K). To investigate whether this control of neuronal activity by APP was related to modification of cholesterol turnover, calcium oscillations were analysed in neurons treated by mevastatin, an inhibitor of HMGCGR. Similar to expression of APP, a 30 min treatment with 12.5 μM
memvastatin completely inhibited spontaneous calcium oscillations in 86 ± 13% of cortical neurons (214 cells analysed in five different experiments; Fig 7L). Interestingly, apamin, a specific antagonist of SK channels, was able to rescue synchronous calcium oscillations in 62 ± 6% of APP expressing neurons (117 cells analysed in three different experiments; Fig 7M) and in 65 ± 19% of neurons treated by mevastatin (112 cells analyzed in three different experiments; Fig 7N). Since the content of membrane cholesterol was not affected by APP (Fig 7B), inhibition of calcium oscillations in APP expressing neurons did not result from alteration of membrane cholesterol. Therefore, we investigated whether geranylgeraniol, the second end product of the mevalonate pathway (Fig 7A), could be involved in the generation of calcium oscillations. Indeed, a two round addition of geranylgeraniol, partially reversed inhibition of calcium oscillations induced by APP (Fig 7O) and mevastatin (Fig 7P) in 38 ± 7% of cortical neurons (195 cells analysed in four different experiments). Altogether, these results indicate that inhibition of cholesterol turnover profoundly affects neuronal activity by activating SK channels. We conclude that APP is able to control neuronal cholesterol turnover needed for neuronal activity.

**DISCUSSION**

Our findings demonstrate that expression of APP at moderate levels in rat cortical neurons decreases cholesterol biosynthesis as well as mRNA levels of several genes involved in cholesterol homeostasis, namely the three SREBP isoforms SREBP1a, SREBP1c, SREBP2, as well as HMGCR, HMGCS and LDLR, which are three SREBP target genes. On the contrary, acute down regulation of endogenous APP expression in cortical neurons increases SREBP1, HMGCR mRNA levels and cholesterol biosynthesis. Among SREBP isoforms, SREBP1 and SREBP2 promote fatty acid and cholesterol synthesis (Brown & Goldstein, 1997). Since SREBP2 was undetectable in our neuronal cultures but SREBP1 has been detected in rodent brain (Ong et al, 2000), we analysed whether APP could specifically alter neuronal processing of SREBP1. We found that in neurons, interaction between APP and SREBP1 in the Golgi inhibits the S2P-mediated release of mSREBP1 and its nuclear translocation, without affecting the processing of ATF6, another substrate of S2P. In contrast, although astrocytes play a central role in the synthesis and metabolism of lipids (Herz, 2001), neither transcription of the HMGCR gene nor biosynthesis of cholesterol were affected by APP in primary cultures of rat cortical astrocytes. We found that APP did not affect production of mSREBP1 and its nuclear translocation because SREBP1 and APP do not interact in these cells. APP also controls SREBP1 expression in vivo. In transgenic mice expressing different APP mutants, down regulation of SREBP1 was observed in brain independently of APP mutations and human PS1 expression. In human brain, APP inversely correlates with SREBP1, including in an AD case with microduplication of APP locus. Recently, mRNA levels of SREBP2 were shown to be decreased in peripheral blood mononuclear cells from AD patients (Mandas et al, 2012).

We also provide evidence that in neurons, APP controls cholesterol turnover by regulating both HMGCR and cholesterol 24-hydroxylase mRNA levels. Decrease in neuronal cholesterol turnover inhibits neuronal activity by activating calcium-dependent potassium SK channels.

Numerous studies have reported that modification of cholesterol content can affect APP processing. High cholesterol level favours production of Aβ (Bodovitz & Klein, 1996; Ehehalt et al, 2003; Schneider et al, 2006), by decreasing the non-amyloidogenic a-secretase activity (Bodovitz & Klein, 1996; Ehehalt et al, 2003; Schneider et al, 2006) and increasing amyloidogenic β- and γ-secretases activities (Grimm et al, 2008; Runz et al, 2002; Yao & Papadopoulous, 2002). In animal models, cholesterol also favors formation of amyloid plaques (Kuo et al, 1998; Refolo et al, 2000; Sparks et al, 1994), while cholesterol depletion reduces amyloid production and its pathological consequences (Fassbender et al, 2001; Grimm et al, 2008; Refolo et al, 2001; Simons et al, 1998). In turn, APP cleavage products, i.e. Aβ and AICD, have been demonstrated to modulate lipid homeostasis (Grimm et al, 2012). In particular, γ-secretase processing of APP has been reported to decrease cholesterol synthesis via inhibition of HMGCR activity and Aβ to impair SREBP2 processing (Grimm et al, 2005; Mohamed et al, 2012).

**Figure 5. APP does not affect SREBP-mediated cholesterol synthesis in astrocytes.** Source data is available for this figure in the Supporting Information.
Figure 5.
Here, we demonstrate that expression of full-length APP decreases HMGCR gene transcription in primary cultures of rat cortical neurons. This regulation of HMGCR gene transcription by APP is however independent of γ-secretase processing, as it was neither unchanged by APPΔC nor inhibited by DAPT. Inhibition of the γ-cleavage of APP prevents the production of AICD, a transcriptional regulator (Huysseune et al, 2009), which inhibits transcription of the LRP1 gene following nuclear translocation and interaction with the LRP1 gene promoter (Liu et al, 2007), but expression of APP without AICD (APPΔC) and inhibition of the γ-cleavage of APP both decreased transcription of the HMGCR gene, indicating that APP controls the transcription by another AICD-independent mechanism controlled by SREBP.

The APP juxta-/transmembrane domain is able to homodimerize and hetero-dimerize with other membrane proteins such as LRP1 and Notch receptors (Kinoshita et al, 2001; Oh et al, 2005; Sato et al, 2009). Not only did APP and SREBP1 co-localize in the Golgi but co-immunoprecipitation demonstrated their association through the APP juxta-/transmembrane domain, containing a GXXXG motif, which plays a key role in the control of SREBP1 and HMGCR expression. Structural analysis recently proposed that this motif in the transmembrane domain of APP is essential to cholesterol binding (Barrett et al, 2012), and in particular the second G of the GXXXG motif, which is conserved in APLPs (Aydin et al, 2012). APLP1 was able to compensate absence of APP in APP knockout neurons and APLP1 and 2 co-immunoprecipitated with SREBP1 (Supporting Information Fig 7). It was previously demonstrated that the C-terminal domain of APP, and most likely full-length APP, can form specific complexes with cholesterol at physiologically relevant cholesterol concentrations (Beel et al, 2008). Whether, in addition to SCAP, APP might function as neuronal cholesterol sensors and/or cargo proteins required for shuttling SREBP1 between ER and Golgi deserves further investigations.
Although C99 and G625L/G629L C99 mutant both contain the signal peptide of APP at their N-terminus and are therefore transported in the secretory pathway (Kienlen-Campard et al, 2008), only C99 was able to mediate SREBP1-dependent regulation of HMGCR expression, indicating the specificity of C99:SREBP1 interaction, which was further confirmed by absence of interaction between APP and ATF6, another substrate of S2P. Reduction of ATF6 expression in APP expressing neurons could be related to a direct SREBP2:ATF6 interaction allowing ATF6, by recruiting HDAC1 to the ATF6-SREBP2 complex, to antagonize SREBP2 (Zeng et al, 2004). Consequently, down regulation of ATF6 expression by APP could be considered as a retro control of expression of SREBP2 target genes.

In vivo, cortical neurons form oscillating networks of various sizes involved in temporal representation and long-term consolidation of information (Buzsaki & Draguhn, 2004). In neuronal networks in culture, control of neuronal cholesterol turnover by APP had important consequences on neuronal activity. In APP expressing neuronal networks, activation of SK channels was involved in the inhibition of calcium oscillations induced by alteration of cholesterol turnover. It was recently demonstrated (Campia et al, 2012) that enhancing the activity of the mevalonate pathway in cardiomyocytes increases the cellular levels of ubiquinone, and the synthesis of ATP, which can inhibit SK channels (Jiang & Haddad, 1997). A decrease in ATP, resulting from inhibition of the mevalonate pathway, could significantly activate SK channels, which have to be inhibited by apamin to rescue calcium oscillations. Interestingly, our preliminary data indicate a significant decrease in ATP level in APP expressing neurons.

In conclusion, our data identify a novel role of APP in neurons, i.e. control of cholesterol biosynthesis and hydroxylation and consequently cholesterol turnover that is crucial for synaptic function. Synaptic activity is regulated by small GTPases proteins (Schubert & Dotti, 2007), and the isoprenoids farnesyl-PP and geranylgeranyl-PP serve as substrates for their prenylation (Hooff et al, 2010). We provide evidence that geranylgeraniol, an end product of the mevalonate pathway, is able to rescue APP-mediated inhibition of synaptic activity. Interestingly, alteration of neuronal cholesterol turnover in CYP46A1 knockout mice induces defects in memory and LTP induction and maintenance (Kotti et al, 2006). In both CYP46A1 knockout mice and APP expressing neurons, production of 24S-hydroxycholesterol, the major neuronal agonist of LXR receptors, is inhibited. Synaptic failure in AD is well established (Selkoe, 2002), and our results argue for stimulation of cholesterol turnover to rescue APP-mediated inhibition of neuronal activity. Such stimulation could be performed by activation of LXR receptors, which could favour neuronal cholesterol synthesis and efflux (Cao et al, 2007). In addition, retinoid X receptor activation was recently demonstrated to stimulate Aβ clearance in an ApoE-dependent manner (Cramer et al, 2012). Together these data indicate the relevance of this novel identified function of APP in neurons, and underline the need to further assess its pathological significance and therapeutic potential.

MATERIALS AND METHODS

Animals and tissues samples
All animal procedures used in the study were carried out in accordance with institutional and European guidelines as certified by Animal Ethics Committee. 5xFAD mice (Oakley et al, 2006) were obtained from Jackson Laboratories (strain: B6SJ-Tg (APPSwFlmJ, PSEN119M146L1286V) 6799Vas/Mmjax) and backcrossed to C57Bl6/J wild type (Wt) mice. C57Bl6/J Wt mice were used as control animals. Experiments on 5xFAD mice and age-matched control were performed at 15 months of age. One hemisphere was frozen in liquid nitrogen and stored at −80°C for biochemical analysis. Brains from 5-month-old wild type and transgenic APP mice (TgAPP expressing hAPP 695 isoform carrying the London mutation) were excised, frozen in liquid nitrogen, and stored at −80°C until analysis. Samples of TgAPP mice (Moehrs et al, 1999) were obtained from reMYND NV (spin-off University Leuven, Belgium).

Postmortem human brains (n = 8) were collected with the approval of the Ethical Committee at the Medical School of the Free University of Brussels. One hemisphere was frozen in liquid nitrogen and stored at −80°C for biochemical analysis. The other hemisphere was fixed by immersion in 10% formalin (v/v) for neuropathological analysis. Postmortem frozen temporal brain tissue from an autosomal dominant early-onset AD patient with APP microduplication was obtained from the Department of Pathology (Rouen University Hospital, France). Genetic analyses, clinical and neuropathological evaluations were previously reported (Cabrero et al, 2006; Rovelet-Lecrux et al, 2006). Briefly, 1 cm coronal sections were performed on the right cerebral hemisphere at the time of autopsy, and immediately frozen at −80°C until use. Neuropathological examination was performed on multiple formalin fixed, paraffin embedded brain tissue samples. This case was characterized by severe amyloid angiopathy and AD lesions corresponding to Braak stage V–VI (Braak & Braak, 1991). In this family, the microduplication corresponded to a 780 kb segment mapping to chromosome 21q2.1, including the APP locus with no contiguous gene.

Reagents and antibodies
All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Antibodies were purchased as indicated: mouse monoclonal WO-2 anti-hAPP and rabbit polyclonal anti-APLP1 and APLP2 antibodies (Millipore, Billerica, MA); rabbit polyclonal anti-APP C-terminus and β-actin (Sigma–Aldrich, St-Louis, MO); anti-SREBP1 N-terminus H-160 (Santa Cruz Biotechnology, La Jolla, CA); anti-KDEL-bearing proteins, TGN46, GM130, golgin 97, SCAP K-19 and GRP78 (Abcam, Cambridge, UK).

Cell cultures
Primary cultures of cortical neurons were prepared from 17 to 18-day-old Wistar rat embryos. Cells were plated in culture dishes (4 × 10⁵ cells/cm²) pre-treated with 10 μg/ml poly-L-lysine in phosphate buffered saline (PBS) and cultured for 6 days in vitro in neurobasal medium supplemented with 2% v/v B-27 medium and 0.5 mM γ-glutamine prior to infection with recombinant adenoviruses. The cultures were maintained at 37°C in a 5% CO₂ atmosphere. Under these conditions, neuronal cultures contain more than 90% neuronal cells, which display high differentiation and survival rate (Brewer, 1995).
Figure 7.
Primary cultures of cortical neurons were prepared from P0–P1 newborn wild type and APP−/− mice. Cortices were dissected in neurobasal medium supplemented with 2% v/v B-27 medium, 0.5 mM l-glutamine and penicillin-streptomycin (50 μg/ml of each), and immediately digested with a trypsin solution (220 μg/ml) containing DNase (1 mg/ml) at 37°C for exactly 3 min. After removal of the trypsin/DNase solution, cortices were further dissociated in neurobasal medium supplemented with DNase (0.5 mg/ml). Cells were plated (4 × 10⁵ cells/cm²) and cultured for 6 days in vitro prior to infection with recombinant adenoviruses (Ad).

Primary cultures of cortical astrocytes were prepared from newborn Wistar rats as previously described (Vermeiren et al., 2005). Briefly, cortices were isolated and dissociated in Dulbecco’s modified Eagle medium (glutaMAX) supplemented with 10% foetal calf serum (FCS), and cortices were isolated and dissociated in Dulbecco’s modified Eagle medium (glutaMAX) supplemented with 10% foetal calf serum (FCS), proline (50 μg/ml), penicillin-streptomycin (50 μg/ml) and fungizone (2.5 μg/ml). After centrifugation, cells were seeded into 75 cm² culture flasks and grown at 37°C in a 5% CO₂ atmosphere. After 7 days, oligodendrocytes were eliminated by shaking. Three days later, cells were plated at 10⁶ cells/cm² in culture dishes pre-treated with 10 μg/ml poly-γ-lysine in PBS. After 2 days, cell differentiation was initiated by decreasing FCS concentration to 3%.

Recombinant adenoviruses and infection

Recombinant adenoviruses encoding β-galactosidase (Adβgal), human APP695 without its C-terminal region (AdAPPΔC) or human APP carrying the Swedish mutation (AdAPPsw) were described previously (Lemarchand et al, 1992). The APPΔC recombinant adenovirus construct contains a stop codon after the three lysine residues that follow the transmembrane domain. Wild-type human APP695 (AdAPP) and AdC99 were constructed and purified using the AdEasy™ XL Adenoviral Vector System (Stratagene, La Jolla, CA). The C99 recombinant adenovirus construct corresponds to the β-cleared C-terminal fragment of hAPP fused to the APP signal peptide sequence. The C99 recombinant lentivirus harbouring the G625SL/G629L mutations was constructed as previously described (Kienlen-Campard et al, 2008). After 6–7 days in culture, cells were infected at a multiplicity of infection of 10 in a minimal volume of culture medium for 4 h. Then, infection medium was replaced by fresh culture medium for 4 days. In these conditions, the recombinant viruses infected about 90% of cells.

Two different plasmids encoding shRNA raised against APP mRNA (APPshRNA1 and APPshRNA2) were obtained from Sigma–Aldrich (St-Louis, MO) and used for construction of recombinant lentiviruses, as previously described (Salmon & Trono, 2007). After 7 days in vitro, APP expression was down-regulated in neurons by infection with lentivirus for 3 days before analysis. In experiments using shRNA lentiviruses, a recombinant lentivirus encoding the neomycin resistance gene was used as a negative control.

**Treatments**

Four days after infection, cells were treated for 8 h with 250 nM DAPT, a functional γ-secretase inhibitor (a kind gift from L. Mercken, Aventis, Paris, France) or for 12 h with 12.5 μM mevastatin, a HMGCR inhibitor (compactin, Sigma–Aldrich, St-Louis, MO). A 100 μM stock solution of compactin was prepared exactly as previously described (Kita et al, 1980).

For cytosolic free Ca²⁺ measurement, acute treatment of neurons with 200 nM apamin (Sigma–Aldrich; St-Louis, MO) was performed using perfusion of the incubation chamber. Rescue of calcium oscillations by geranylgeraniol (Sigma–Aldrich, St-Louis, MO) was performed at 2 mM, respectively. Geranylgeraniol was first dissolved in a small volume of ethanol as previously described (Kotti et al, 2008).

**Aβ measurements**

Human and rodent Aβ40 were determined in the same cell culture supernatants using the Multi-Spot Human and Rodent (6E10 and 4G8, respectively) Aβ Triplex Assay and theSECTOR Imager 2400 (Meso Scale Discovery) according to manufacturer’s instructions.

**Cholesterol extraction and assay**

Cholesterol was extracted and purified as previously described (Blight & Dyer, 1959). Briefly, 4 days after infection, cells were harvested in water and lipids were extracted with 3 volumes of chloroform/methanol (2:1 v/v), stirred 1 min and centrifuged at 1760 g for 15 min.

**Figure 7. Inhibition of mevalonate pathway by APP impairs neuronal activity.** Source data is available for this figure in the Supporting Information.
The organic phase was collected and washed with 2 volumes of 0.05 M NaCl, then twice with 2 volumes of 0.36 M CaCl₂/methanol solution (1:1 v/v) and centrifuged at 1,760 g for 15 min. Organic phase was collected, dried under argon, and harvested in 0.5% Triton X-100.

Cholesterol was assayed using the cholesterol oxidase-base Amplex Red Cholesterol Assay kit (Molecular Probes™, Invitrogen) and cholesterol esterase was omitted from the assay to exclude intracellular cholesterol esters.

**Cholesterol synthesis**

Cholesterol synthesis was measured 4 days after infection. Cells were washed with PBS and [1-14C] acetate (2.5 μCi in 0.5 ml) was added for 4 h. After washing, cells were scraped, collected at 150 g for 5 min, resuspended in PBS (200 μl) and solubilized by addition of 20 volumes of chloroform/methanol (2:1 v/v). Four volumes of 0.9% NaCl were added to separate the two phases. The chloroform phase was dried under a nitrogen flux, hydrolyzed for 1 h at 80 °C in 1 ml of methanol containing 1 M KOH, then hexane was added for extraction. The upper phase was collected, dried and analysed by thin layer chromatography using silica gel 60 plate (Merck, Darmstadt, Germany), which was developed with hexane/diethyl ether/acetic acid (87:20:1 by volume), with reference to internal cholesterol standard (Sigma, St Louis, MO). Radioactivity was quantified using an Instant Imager (PerkinElmer, Waltham, MA) and standards were visualized in iodine vapours. The radioactivity was further measured in a β-counter. Results were normalized to total protein content.

**Western blotting**

Cells lysate (10 μg protein) were analysed by Western blotting using 4–12% Nupage™ bis–Tris gels (Invitrogen). Nitrocellulose membranes were incubated overnight at 4°C with the following primary antibodies: human APP-specific WO-2 (1:2,000); anti-APP C-terminal (1:2,000); anti-N-terminus of SREBP1 H-160 (1:1,000); anti-APLP1 (1:4,000); anti-APLP2 (1:2,000); anti-SCAP K-19 (1:1,000); anti-GRP78 (1:1,000); anti-β-actin (1:2,000) and anti-ATF6 (1:1,000). Blots were incubated with horseradish peroxidase-conjugated secondary antibodies, revealed by ECL (Amersham Pharmacia), and quantified using the Quantity One™ software (Bio-Rad Laboratories, Hercules, CA). Actin was used as internal standard to normalize protein load in gels.

**Subcellular fractionation**

Cells were harvested on ice in 0.25 M sucrose containing 1 mM EDTA, 3 mM imidazole buffered at pH 7. Cell suspension was homogenized in a tight Dounce homogenizer. A low-speed nuclear fraction was pelleted at 1,000 g for 10 min and extracted ten times by resuspension and sedimentation. A high-speed membrane fraction of poled postnuclear supernatants was further sedimented at 100,000 g for 60 min in a Ti50 rotor (Beckman). Nuclear and membrane fractions were analysed by Western blotting using 4–12% Nupage™ gels as above.

**Immunoprecipitation**

Neurons or astrocytes were scraped and pelleted in cold PBS. Cells were solubilized in immunoprecipitation buffer (25 mM Tris pH 6.8, 0.5% Triton X-100, 0.5% Nonidet P-40) containing a protease inhibitor cocktail (Calbiochem, San Diego, CA). Samples were immunoprecipitated (500 μg of proteins) with 2 μg H-160, WO-2 or SCAP K19 antibodies overnight at 4°C. Protein A-sepharose (50 mg/ml, Amersham Pharmacia) was then added for 4 h. The samples were centrifuged at 15,800 g for 2 min at 4°C, and pellets were washed twice with immunoprecipitation buffer and once with TBS buffer (10 mM Tris, pH 7.5). Pellets were incubated for 5 min at 95°C in sample buffer (125 mM Tris pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 1% bromophenol blue). Samples were centrifuged at 15,800 g for 2 min at 4°C and supernatants were analysed by Western blotting using 4–12% Nupage™ gels as above.

**Immunofluorescence**

Neurons or astrocytes were seeded at 10⁵ cells/cm² on glass coverslips, fixed with 4% w/v formaldehyde at room temperature then permeabilized with 1% Triton X100 (v/v) in PBS both for 15 min. Non-specific binding was prevented by 1 h preincubation in PBS/3% non-fat dry milk, followed by 1 h incubation with primary antibodies: H-160 (1:100); WO-2 (1:100); anti-GM130 (1:50), anti-TGN46 (1:50) and anti-KDEL (1:50). After three washes with PBS, samples were incubated for 1 h with 5 μg/ml Alexa-labelled secondary antibodies (Molecular Probes, Invitrogen). After three additional washes, preparations were mounted in Moviol and examined with a LSM 510 META confocal microscope (Zeiss, Jena, Germany) using a Plan-Apochromat 63X/1.4 oil DIC objective.

**RNA extraction and real time PCR**

Total RNA was isolated by TriPure Isolation Reagent according to the manufacturer’s protocol. RNA samples were resuspended in DEPC-treated water. Reverse transcription was carried out with the iScript cDNA synthesis Kit, using 1 μg of total RNA in a total volume reaction of 20 μl. Controls were performed without reverse transcriptase to rule out amplification of contaminant genomic DNA. Real-time PCR was performed for the amplification of HMGCR and glyceraldehyde phosphate dehydrogenase (GAPDH) cDNAs. Primers were purchased from Sigma–Aldrich (St-Louis, MO); F, Forward primer; R, Reverse primer.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>F-5’</td>
<td>-TGGCTGCTTGTCTGCTCTG-3’</td>
</tr>
<tr>
<td>R-5’</td>
<td>-CACAC-AATTCCGGCAAGCT-3’</td>
</tr>
<tr>
<td>CYP46A1</td>
<td>-TGAGCTGCTTCTGCTGCTG-3’</td>
</tr>
<tr>
<td>R-5’</td>
<td>-TTGCTGTTGAGGTACTGTA-3’</td>
</tr>
<tr>
<td>F-5’</td>
<td>-CGCTAAGAATGTTGCTTCCG-3’</td>
</tr>
<tr>
<td>R-5’</td>
<td>-GGCTAAGAATGTTGCTTCCG-3’</td>
</tr>
<tr>
<td>BIP/Grp78</td>
<td>-TTGTGTTGAGGTACTGTA-3’</td>
</tr>
<tr>
<td>F-5’</td>
<td>-TGGCTGCTTGTCTGCTCTG-3’</td>
</tr>
<tr>
<td>R-5’</td>
<td>-TTGCTGCTTGTCTGCTCTG-3’</td>
</tr>
<tr>
<td>SERCA2</td>
<td>-ACGAGGCA-GGCGATGTTTTC-3’</td>
</tr>
<tr>
<td>R-5’</td>
<td>-ACGAGGCA-GGCGATGTTTTC-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>-CCCCCAATGTACCGTCAGCT-3’</td>
</tr>
<tr>
<td>R-5’</td>
<td>-CCCCCAATGTACCGTCAGCT-3’</td>
</tr>
</tbody>
</table>

Primers used for the amplification of SREBP1a, SREBP1c and SREBP2 cDNAs were kindly obtained from E. Lefai, INSERM, Lyon, France (Gosmain et al, 2005).
The paper explained

PROBLEM:
Alzheimer disease (AD) is characterized by the presence in the brain of two types of lesions corresponding to intraneuronal neurofibrillary tangles, and extracellular senile plaques, containing an amyloid deposit of Aβ peptide produced from the APP. The amyloid cascade hypothesis assumes that Aβ plaques cause dementia. However, anti-Aβ therapies in humans have, so far, failed. Although processing of APP has been extensively studied, the neuronal function of the protein remains unclear. GWAS on AD recently confirmed that the epsilon 4 allele of the ApoE gene is a major risk factor and provided evidence for other risk genes encoding proteins involved in cholesterol homeostasis. These susceptibility loci further support the hypothesis that perturbation of lipids metabolism favours progression of AD. We studied whether APP is able to control cholesterol homeostasis.

RESULTS:
The biosynthesis of cholesterol is regulated by SREBP. In cultured neurons, interaction between APP and SREBP controls production the mature SREBP transcription factor and consequently expression of its target genes, including the gene encoding HMGCR, the limiting enzyme in biosynthesis of cholesterol. Therefore, expression of APP inhibits biosynthesis of cholesterol while down regulation of endogenous APP has the opposite effect. However, APP, under conditions of excess or defect, does not affect neuronal membrane cholesterol content. This results from the APP-mediated control of the expression of cholesterol 24-hydroxylase, a neuronal specific enzyme involved in hydroxylation of cholesterol. Consequently, APP controls cholesterol turnover. Such a regulation does not occur in astrocytes, in which APP and SREBP do not interact.

The control of neuronal cholesterol turnover by APP in the mevalonate pathway has important consequences on neuronal activity. APP and mevastatin both reduce cholesterol synthesis and turnover, leading to inhibition of neuronal activity by activation of calcium-dependent potassium SK channels. Apamin, a specific antagonist of SK channels, and geranylgeraniol produced in the mevalonate pathway, are able to rescue neuronal activity in both hAPP expressing and mevastatin treated neurons.

IMPACT:
Our study provides new insight in an important function of APP, which is able to control neuronal cholesterol turnover needed for neuronal activity. These findings have important clinical applications, as they offer therapeutical alternatives for AD treatment based on the function rather than the processing of APP. Cholesterol turnover can be stimulated by activation of nuclear receptors of the liver X receptor (LXR) family, which are activated by oxysterols, and in particular by 24-S-hydroxycholesterol produced in neurons under the control of APP. Such activation could improve the production of end products generated in the mevalonate pathway in order to prevent synaptic dysfunctions underlying learning and memory deficits observed in AD. Activation of RXR was recently demonstrated to induce ApoE-mediated clearance of Aβ and to reverse deficits in a mouse model of AD, further emphasizing the stimulation of neuronal cholesterol turnover as a possible target for the treatment of dementia.

Real-time PCR was carried out in a total volume of 25 µl containing 2 ng cDNA template, 0.3 µM of the appropriate primers and the IQ™ SYBR® Green Supermix 1×. The PCR protocol consisted of 40 amplification cycles (95°C for 30 s, 60°C for 45 s and 79°C for 15 s) and was performed using an iCycler IQ™ multicolor Real-Time PCR detection system (Bio-Rad). For quantification, a relative standard curve was determined in the same conditions for each target gene with a fourfold dilution (from 100 to 0.097 ng) of a cDNA template mix. Each sample was normalized with the relative expression of GAPDH. Calculation of Cn, standard curve preparation and quantification of mRNA in the samples were performed by the ‘post run data analysis’ software provided with the iCycler system (Bio-Rad).

Cytosolic free Ca²⁺ measurement in single neurons
Neurons were plated at a density of 1.8 × 10⁵ cells/cm² on coated 22 mm round glass coverslips. Four days after adenoviral infection, neurons were incubated in the dark in the presence of the Ca²⁺ indicator fura-2 acetoxyxymester (Fura-2 AM; Calbiochem, Camarillo, CA, USA) at a final concentration of 2 µM in Krebs-HEPES buffer (10 mM HEPES, 135 mM NaCl, 6 mM KCl, 2 mM CaCl₂, 1.2 mM MgCl₂, 10 mM glucose, pH 7.4) for 30 min at room temperature. Coverslips were then washed and mounted in a heated (37°C) microscope chamber (1 ml). Cells were alternately excited (1 or 2 Hz) at 340 and 380 nm for 100 ms using a Lambda DG-4 Ultra High Speed Wavelength Switcher (Sutter Instrument, Novato, CA) coupled to a Zeiss Axiovert 200 M inverted microscope (X20 fluorescence objective; Zeiss Belgium, Zaventem, BE). Images were acquired with a Zeiss Axioimam camera coupled to a 510 nm emission filter and analysed with the Axiovision software. Calcium concentration was evaluated from the ratio of fluorescence emission intensities excited at the two wavelengths. Baseline mean ratio value (Rmean) was the mean ratio value after recording. Calcium oscillations expressed as Rmean were defined as variations of more than 10% from Rmean, occurring synchronously in several cells of the field.

Statistical analysis and presentation of the results
The number of samples (n) in each experimental condition is indicated in figure legends. An unpaired Student’s t test is applied when the statistical units underlying two samples being compared are non-overlapping and a one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple-comparison post-test is applied when many subgroups are compared. Differences were considered significant at
and JNO wrote the manuscript with critical evaluation by ID, designed peptides. All authors critically discussed results. NP AL, DC and JPB provided samples of Alzheimer’s patients, JBD of shRNA on astrocytes, FK performed subcellular fractionation; t-experiments and A

Author contributions
NP and JNO designed research; NP performed primary cultures, infection and biochemical experiments, production of shRNA and viruses, calcium oscillations analysis; NP and DT performed immunofluorescence; LA conducted measurements of cholesterol synthesis; ID provided 5XFAD mice; PG provided inverted microscope for calcium imaging; AH provided APP knockout and viruses, calcium oscillations analysis; NP and DT performed

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Supporting Information is available at EMBO Molecular Medicine Online

The authors declare that they have no conflict of interest.

For more information

References

p < 0.05 (‘p < 0.1, “p < 0.01; ‘’p < 0.001). Each Western blot shown is representative of at least three independent experiments. For LTP experiments and Aβ assay, statistical analysis was done with Student’s t-test on fEPSP slope values post stimulation.

Research Article
Nathalie Pierrot et al.


Johnston J, O’Neill C, Lannfelt L, Winblad B, Cowburn RF (1994) The region of the yeast KAR2 (BiP) gene contains a regulatory domain that responds to the presence of unfolded proteins in the endoplasmic reticulum


Kotti T, Head DD, McKenna CE, Russell DW (2008) Biphasic requirement for geranylgeranylation in hippocampal long-term potentiation. Proc Natl Acad Sci USA 105: 11394-11399


Lund EG, Guileyardo JM, Russell DW (1999) DNA cloning of cholesterol metabolism-related gene expression in peripheral blood mononuclear cells from Alzheimer patients. Lipids Health Dis 11: 39


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