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Cholesterol 24S-Hydroxylase Overexpression Inhibits the Liver X Receptor (LXR) Pathway by Activating Small Guanosine Triphosphate-Binding Proteins (sGTPases) in Neuronal Cells

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Abstract The neuronal-specific cholesterol 24S-hydroxylase (CYP46A1) is important for brain cholesterol elimination. *Cyp46a1* null mice exhibit severe deficiencies in learning and hippocampal long-term potentiation, suggested to be caused by a decrease in isoprenoid intermediates of the mevalonate pathway. Conversely, transgenic mice overexpressing CYP46A1 show an improved cognitive function. These results raised the question of whether CYP46A1 expression can modulate the activity of proteins that are crucial for neuronal function, namely of isoprenylated small guanosine triphosphate-binding proteins (sGTPases). Our results show that CYP46A1 overexpression in SH-SY5Y neuroblastoma cells and in primary cultures of rat cortical neurons leads to an increase in 3-hydroxy-3-methyl-glutaryl-CoA reductase activity and to an overall increase in membrane levels of RhoA, Rac1, Cdc42 and Rab8. This increase is accompanied by a specific increase in RhoA activation. Interestingly, treatment with lovastatin or a geranylgeranyltransferase-I inhibitor abolished the CYP46A1 effect. The CYP46A1-mediated increase in sGTPases membrane abundance was confirmed in vivo, in membrane fractions obtained from transgenic mice overexpressing this enzyme. Moreover, CYP46A1 overexpression leads to a decrease in the liver X receptor (LXR) transcriptional activity and in the mRNA levels of ATP-binding cassette transporter 1, sub-family A, member 1 and apolipoprotein E. This effect was abolished by inhibition of prenylation or by co-transfection of a RhoA dominant-negative mutant. Our results suggest a novel regulatory axis in neurons; under conditions of membrane cholesterol reduction by increased CYP46A1 expression, neurons increase isoprenoid synthesis and sGTPase prenylation. This leads to a reduction in LXR activity, and consequently to a decrease in the expression of LXR target genes.

Keywords CYP46A1 · Cytochrome P450 · Brain metabolism · Cholesterol · Small GTPases · Isoprenoids · Prenylation · RhoA

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Introduction

Cholesterol, apart from being an essential component of cellular membranes, is a key regulator of cellular processes in the central nervous system (CNS), playing a role in promoting

neurite outgrowth [1–5], in synaptogenesis and synaptic integrity, and in neurotransmitter release [6–9]. In addition to the brain-specific activities, cholesterol synthesis generates intermediate products, such as isoprenoids and ubiquinone [10]. Therefore, constant levels of this sterol are required for normal brain functioning. Its homeostasis is maintained, in part, by an efficient blood–brain barrier that prevents exchanges with lipoprotein cholesterol from circulation. Thus to meet cholesterol needs, de novo and in situ synthesis occur in the CNS. The conversion of cholesterol into 24(*S*)-hydroxycholesterol (24OHC), by the neuronal-specific cytochrome P450 cholesterol 24S-hydroxylase (CYP46A1), has been described as the major cholesterol elimination mechanism [11–13]. In vitro, 24OHC has been shown to be a ligand for the liver X receptor (LXR) [14, 15]. Surprisingly, however, in transgenic mice overexpressing CYP46A1, activation of the LXR pathway was not observed, despite the higher levels of 24OHC [16]. In accordance to this, selective overexpression of CYP46A1, in the cortex and hippocampus of transgenic mice model for AD, ameliorated the amyloid β pathology, without activation of the LXR pathway [17]. Knockout mice lacking *Cyp46a1* have a≈40 % reduction in brain cholesterol excretion, but this decrease is compensated for by a reduction in the de novo synthesis that maintains normal steady-state levels [13]. *Cyp46a1* null mice exhibit severe deficiencies in spatial, associative and motor learning, and hippocampal long-term potentiation (LTP) [18, 19]. Interestingly, these studies with knockout mice are consistent with the possibility that a decrease in cholesterol synthesis may limit the supply of isoprenoid intermediates of the mevalonate pathway, required for learning and synaptic plasticity. Accordingly, transgenic mice overexpressing CYP46A1 exhibit increased brain cholesterol synthesis, increased levels of synaptic proteins in the hippocampus, as well as improved memory function when compared to wild-type controls [16, 20].

These results have raised the question of whether CYP46A1 expression levels may alter the availability of isoprenoids intermediates, possibly affecting the amount of crucial isoprenylated proteins in neurons. Indeed, mevalonate, the product of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), can be converted into farnesyl-diphosphate (FFP) and geranylgeranyl-diphosphate (GGPP) that, among other functions, can covalently bind to proteins, such as most small guanosine triphosphate-binding proteins (sGTPases), and act as anchors for membrane association. sGTPases comprise a superfamily of monomeric proteins classified into, at least, five families (Ras, Rho, Rab, Sar1/Arf, and Ran) that are involved in complex signaling networks and regulate diverse cellular activities. These activities include intracellular vesicle transport, cell adhesion, endocytosis, cytoskeletal organization, cell cycle progression, receptor signaling, vesicle trafficking, and gene expression [21, 22]. Membrane association and targeting, binding to regulators, and activation of downstream effectors are dependent on lipid post-translational modifications for all sGTPases, except those from the Ran family [21]. Sar/Arf sGTPases undergo post-translational myristoylation, while the Ras, Rho, and Rab families undergo a C-terminal prenylation. While Ras proteins are farnesylated [23, 24], Rho proteins are geranylgeranylated or farnesylated [25], and Rab proteins are mainly di-geranylgeranylated but some can be mono-geranylgeranylated [26, 27]. Prenylated sGTPases cycle between a GDP (inactive)/GTP (active) binding conformation that combines with cytosol/membrane alternation, which is mediated by guanine dissociation inhibitors. These latter inhibitors form soluble complexes with sGTPases by binding their lipid moiety and are responsible for the extraction and delivery of sGTPases to membranes [21, 28].

Rho proteins have been extensively associated with actin cytoskeleton remodeling and are therefore critical to CNS neuronal cell migration, axon growth, guidance and branching, dendritic spine formation and stabilization, growth cone motility and collapse, and synapse formation [29–31]. Additionally, Rho GTPases have also been implicated in the correct formation of myelin sheaths [32]. Ras are involved in axon growth, memory formation, LTP induction and maintenance, and synaptic modulation [31, 33, 34], while Rab proteins have been mainly associated with endocytosis, synaptic vesicles exocytosis, axonal retrograde transport, and postsynaptic compartment dynamics of glutamate receptors [35]. In accordance to their pivotal role in the CNS, Ras, Rho, and Rab sGTPases have also been implicated in a number of neurological diseases. These diseases range from genetic defects such as X-linked non-specific mental retardation, Niemann–Pick disease Type C or choroideremia to sporadic neurological disorders such as late-onset AD, Parkinson's disease, multiple sclerosis, brain tumors, or even brain disorders resulting from ischemic stroke [10, 30, 36–42].

In the present work, evidence is presented that CYP46A1 expression levels are able to modulate isoprenoids synthesis and, ultimately, protein prenylation and activation of sGTPases. This modulation is shown to be important for activation of the LXR signaling system. Our results explain the surprising finding that this system is not activated in CYP46A1 transgenic mice in spite of high levels of the efficient LXR activator 24S-hydroxycholesterol [16].

Materials and Methods

Reagents and Antibodies

The following primary antibodies were used: anti-RhoA (clone 67B9, #2177; Cell Signaling Technology, Beverly, MA, USA), anti-Rac1 (clone 23A8, #05-389; Millipore, Temecula, CA, USA), anti-Rab8 (clone 4/Rab4, #610844; BD

Transduction Laboratories), anti-Cdc42 (clone B-8, sc-8401; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-apolipoprotein E (APOE) (clone M-293, sc-98574; Santa Cruz), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (clone 6C5, sc-32233; Santa Cruz), anti-FLAG®M2 (clone M2, F1804; Sigma-Aldrich Inc., St Louis, MO, USA), anti-calnexin (ab7580; Abcam, Cambridge, UK), and anti-HA-Peroxidase (3F10) (#12013819001, Roche Applied Science). The chemical inhibitors GGTi-2133 and lovastatin were purchased from Sigma and Pefabloc® from Roche.

Cell Culture

The SH-SY5Y human neuroblastoma cell line was cultured as previously described [43]. Briefly, cells were maintained in low glucose DMEM (Sigma), at 37 °C in humidified 5 % CO_2. The media was supplemented with 10 % heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco®, Invitrogen).

Primary cultures of rat cortical neurons were prepared from 17- to 18-day-old fetuses of Wistar rats as described previously [44] with minor modifications. In short, pregnant rats were sacrificed in a CO_2 chamber and the fetuses were collected in Hank's balanced salt solution and rapidly decapitated. After removal of meninges and white matter, the brain cortex was collected in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS-2). The cortex was then mechanically fragmented, transferred to 0.05 % trypsin, and incubated for 15 min at 37 °C. Following trypsinization, cells were washed twice in HBSS-2 containing 10 % fetal bovine serum and resuspended in Neurobasal medium (Gibco®), supplemented with 0.5 mM L-glutamine, 25 μM L-glutamic acid, 2 % B-27 supplement (Gibco®), and 12 mg/mL gentamicin. Isolated neurons were plated with a density around 640/mm^2 on culture plates pre-coated with poly-D-lysine and maintained at 37 °C in a humidified atmosphere of 5 % CO_2. Half of the medium of the neuronal primary cultures was changed every 3–4 days until the 26th day in vitro (26DIV). Glutamic acid was only added into the medium when plating the cells. All the media changes and cell treatments afterwards were free from glutamic acid.

Preparation of Cytosolic and Membrane Fractions and Total Extracts from Cultured Cells

For the isolation of cytosolic and membrane fractions, cells were harvested in ice-cold cell wash buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], and 1 mM of Pefabloc®), and centrifuged at 1,000×*g* for 10 min at 4 °C. The pellet was resuspended in cell homogenisation buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.25 M sucrose, 1 mM DTT, 1× protease inhibitor cocktail-Roche Diagnostics GmbH, Penzberg, Germany) and homogenized by 20 passages through a 23× gauge needle attached to a 1-mL syringe. The homogenate was centrifuged at 10,000×*g* for 5 min and the post-nuclear supernatant obtained was subjected to ultra-centrifugation at 100,000×*g* for 1 h at 4 °C. The supernatant (cytosolic fraction—S100) was collected and the pellet (membrane fraction—P100) was resuspended in homogenization buffer.

For the preparation of total cell extracts, cells were harvested in phosphate-buffered saline and resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 % Triton-X 100) containing 1 mM DTT and 1× protease inhibitor mixture. After incubation at 4 °C, for 30 min, samples were sonicated four times for 4 s each, on ice, followed by centrifugation at 12,000×*g* for 15 min at 4 °C.

Protein concentration was determined by Bradford method and extracts were stored at −80 °C until further use.

Preparation of Cytosolic and Membrane Fractions from Mice Brain Cortex

Brain cortex tissue was obtained from *wild-type* and homozygous HA-tagged CYP46A1 transgenic mice, previously described and characterized [20]. Heterozygous mice cross-bred with C57Bl6 mice [16] from the sixth generation were inbred for an additional three generations in order to obtain the homozygous mice [20]. To isolate the cytosolic and membrane fractions of brain cortex tissue, a slice was cut and placed in ice-cold tissue homogenization buffer (250 mM Sucrose, 100 mM potassium phosphate pH 6.7, 5 mM $MgCl_2$, 1 mM DTT, and 1× protease inhibitor mixture). The tissue was homogenized with a motor-driven Bio-vortexer (NO1083; Biospec Products, Bartlesville, OK, USA) and centrifuged at 3,500×*g* for 10 min at 4 °C. The resulting pellet, containing nuclei and cell debris, was discarded and the supernatant was further centrifuged at 100,000×*g* for 30 min at 4 °C. The supernatant (cytosolic fraction—S100) was collected and the pellet (membrane fraction—P100) was resuspended in homogenization buffer.

Western Blot Analysis

Proteins were subject to SDS-PAGE gels, electroblotted onto Immobilon P membrane (IPVH00010, Millipore), and incubated with specific antibodies. Results were quantified using the Quantity One version densitometry analysis software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

RhoA Activity Assay

RhoA activity was measured in SH-SY5Y cells lysates using an ELISA-based RhoA activation assay Biochem Kit™ (G-LISA™, Cytoskeleton Inc., Denver, CO, USA), according to the manufacturer's instructions. Briefly, fresh cell lysates were added to the RhoA-GTP affinity plate that was coated with the

Rhotekin-binding domain of RhoA for 30 min. The lysates were incubated with primary and secondary antibodies for 45 min each. After adding the detection reagent, the luminescence was measured in GloMax®-Multi Detection System (Promega, Madison, WI, USA).

Expression Analysis

Total cell RNA was extracted using Trizol Reagent (Invitrogen) following manufacturer's instructions. Real-time PCR (qPCR) analysis for HMGR and 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMGS), LDL receptor (LDLR), ATP-binding cassette transporter, sub-family A, member 1 (ABCA1), and APOE was performed using SYBR green Master Mix in an ABI 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA) and specific primers (Table 1). Results presented were obtained from at least three individual experiments and each sample was assayed in triplicate. The mRNA levels of the genes of interest were normalized to the mRNA levels of β-actin and expressed as fold changes relative to controls, using the ΔΔCt method. Statistical analysis was performed with the ΔCt values.

HMG-CoA Reductase Activity Levels

HMGR activity was measured using the HMG-CoA Reductase Assay Kit (Sigma) following manufacturer's instructions. Briefly, the cells were harvested and subjected to a centrifugation of 500×*g*, 5 min at 4 °C. The pellet was resuspended in 1× assay buffer and homogenized by 20 passages through a 25× gauge needle attached to a 2-mL syringe. The cell extract was centrifuged again for 5 min, 500×*g* at 4 °C and the supernatant containing the cellular content was collected. After protein content determination by the Bradford method, 150 μg of cell extract were added to a reaction solution containing 400 μM NADPH, 0.3 mg/mL HMGCR with or without 500 nM pravastatin in a total volume of 200 μL per well in a 96-well plate. The absorbance at 340 nm was subsequently measured every 30 s for 50 min in Infinite® M200 (Tecan, Männedorf, Switzerland). The decrease in absorbance represents the oxidation of NADPH mediated by HMGR. Each sample was assayed in duplicate. The results are expressed as nanomole of NADPH consumed per minute per milligram of protein.

Table 1 List of primers used for qPCR

Gene	Species	Sequence (5′ – 3′) / source
HMGR	Human	5′ ATAGGAGGCTACAACGCCCAT 3′ (fwd)
		5′ TTCTGTGCTGCATCCTGTCC 3′(rev)
HMGS	Human	5′ GGCACAGCTGCTGTCTTCAAT 3′ (fwd)
		5′ ACCAGGGCATACCGTCCAT 3′ (rev)
LDLR	Human	5′ CAGATATCATCAACGAAGC 3′ (fwd)
		5′ CCTCTCACACCAGTTCACTCC 3′ (rev)
APOE	Human	5′ GACTGGCCAATCACAGGC 3′ (fwd)
		5′ CTGTCTCCACCGCTTGCTC 3′ (rev)
	Rat	5′ CTTCTGGATTACCTGCGCT 3′ (fwd)
		5′ GTCCTCCATCAGTACCGTCAG 3′ (rev)
ABCA1	Human	5′ CCTGTTTCCGTTACCCGACTC 3′ (fwd)
		5′ ACAGGCGAGCCACAATGG 3′ (rev)
	Rat	5′ CCCGGCGGAGTAGAAAGG 3′ (fwd)
		5′ AGGGCGATGCAAACAAAGAC 3′ (rev)
β – actin	Human	5′ CTGGAACGGTGAAGGTGACA 3′ (fwd)
		5′ AAGGGACTTCCTGTAACAATCCA 3′ (rev)
	Rat	5′ CTTGCAGCTCCTCCGTCGCC 3′ (fwd)
		5′ CTTGCTCTGGGCCTCGTCGC 3′ (rev)

Gene Reporter Assay

To minimize variations in transfection efficiency, replicates were transfected in single batch suspension with X-tremeGENE HP (Roche), according to the manufacturer's instructions. Plates containing 150,000 cells were co-transfected with 0.5 μg of the reporter plasmid, 0.1 μg of each expression vector, together with 0.025 μg of a β-galactosidase expression plasmid (pSV40-βGAL). Cells were inoculated in 24-well plates and maintained for 48 h. Cells were harvested and lysed in reporter lysis buffer (Promega). Cell extracts were assayed for luciferase and β-galactosidase activity (β-Gal Reporter Gene Assay, Roche).

Total Cholesterol Levels

Total cholesterol was quantified using the Amplex® Red cholesterol assay kit (Invitrogen) according to the manufacturer's instructions. Cell membrane fractions were diluted 1:50 in 1× reaction buffer and 50 μL was used to quantify cholesterol. Samples were placed in a 96-well plate and the reaction was initiated by adding 50 μL of the Amplex® Red reagent/HRP/cholesterol oxidase/cholesterol esterase working solution to each well. The reactions were incubated for 30 min at 37 °C. Fluorescence measurements were performed in a GloMax®-Multi Detection System (Promega). Each sample was assayed in triplicate. Cholesterol levels were normalized with total protein levels and expressed as microgram of cholesterol per milligram of protein.

Statistics

Statistical analysis was performed using the Student's *t* test, Mann–Whitney test, the ANOVA one-way and ANOVA two-way tests followed by Tukey or Dunnet multiple comparisons test. All analysis was performed using the GraphPad Prism

version 5.01 for Windows, GraphPad Software, La Jolla, California, USA, www.graphpad.com.

Results

Overexpression of CYP46A1 Increases sGTPases Prenylation and Activity

We have hypothesized that alterations in CYP46A1 expression levels may affect the availability of isoprenoid intermediates of the mevalonate pathway, thereby influencing the amount of crucial isoprenylated proteins in neuronal cells. Although it has been described that a decrease in cholesterol synthesis affects the supply of isoprenoids in *Cyp46a1* knockout mice [18, 19], it is not known whether this decrease influences protein prenylation, which is a critical post-translational modification for many signalling proteins, namely most sGTPases. Therefore, we started to investigate whether an increase in CYP46A1 expression affects prenylation and activity of members of the Rho and Rab families of sGTPases in two different cellular models, with distinct endogenous CYP46A1 expression levels. We have used primary cultures of rat cortical neurons maintained for 26DIV which express high levels of CYP46A1, and SH-SY5Y human neuroblastoma cells, where CYP46A1 mRNA can barely be detected [43].

Firstly, to determine if the recombinant CYP46A1 protein retains its catalytic activity, we have transfected SH-SY5Y cells and 26DIV neurons with an expression vector coding for the human CYP46A1 (pFLAG-hCYP46A1) for 48 and 24 h, respectively. These time points were chosen based on a time course analysis of transient CYP46A1 protein expression levels (data not shown). As expected, CYP46A1 overexpression induced a significant decrease in membrane cholesterol content. Membrane cholesterol levels decrease by 60 % in SH-SY5Y cells and by 30 % in 26DIV neurons transfected with pFLAG-hCYP46A1 as compared to cells transfected with empty vector (pFLAG) (Fig. 1).

Prenylation is an essential modification for membrane anchoring of sGTPases. Hence, membrane association of sGTPases provides a sensitive measure of their prenylation status [23]. Therefore, we have isolated membrane and cytosolic fractions of SH-SY5Y cells and 26DIV neurons, and the content of RhoA, Rac1, Cdc42, and Rab8 was determined by immunoblotting.

Our results show an overall significant increase in membrane association of all sGTPases tested after CYP46A1 overexpression in SH-SY5Y cells and 26DIV neurons (Fig. 2). In fact, we observed an increase of about 35, 53, 63, and 130 % in the membrane levels of RhoA, Rac1, Cdc42, and Rab 8, respectively, in SH-SY5Y cells transfected for 48 h with

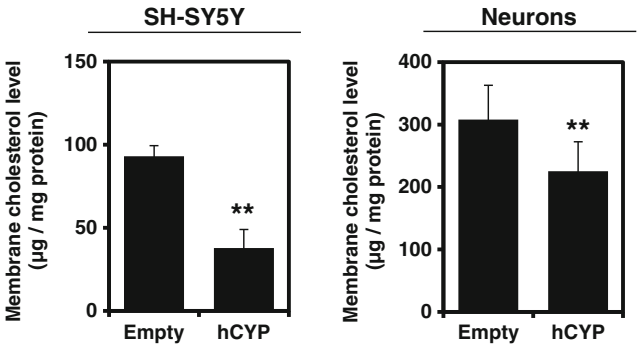

Fig. 1 CYP46A1 overexpression reduces cholesterol content in the membrane fraction of SH-SY5Y and 26DIV neurons. Membrane cholesterol levels were determined with the Amplex®Red cholesterol determination kit in the membrane fraction isolated from SH-SY5Y cells and in 26DIV neurons transfected with empty pFLAG (Empty) or pFLAG-hCYP46A1 (hCYP) for 48 and 24 h, respectively. Data represents mean values±SEM from at least three independent experiments and is expressed as microgram of membrane cholesterol per milligram of membrane protein (**$p<0.01$)

pFLAG-hCYP46A1, as compared to cells transfected with empty vector (Fig. 2a). Concomitantly, we could observe a significant decrease of about 50, 30, 25, and 20 % in the cytosolic levels of RhoA, Rac1, Cdc42, and Rab 8, respectively (Fig. 2b). These results were further confirmed in 26DIV neurons transfected with pFLAG-hCYP46A1 for 24 h, where we could also observe a significant increase of approximately 65, 26, 21, and 97 % in the membrane levels of RhoA, Rac1, Cdc42, and Rab 8, respectively (Fig. 2c) and a concomitant decrease of about 5 % in RhoA and Rac1, and 15 and 25 % in Cdc42 and Rab8, respectively in the cytosolic fraction (Fig. 2d). Importantly, this CYP46A1-dependent increase of membrane-associated sGTPases is not due to an increase in their abundance as the analysis of total cell protein extracts by immunoblotting shows that the total protein levels of the tested sGTPases remain unchanged after overexpression of hCYP46A1 both in neuroblastoma cells and in 26DIV neurons (data not shown).

Overall, these results clearly demonstrate the effective translocation of RhoA, Rac1, Cdc42, and Rab8 from the cytosol to membranes induced by increased CYP46A1 expression.

To assess if the CYP46A1-dependent increase in the membrane association of sGTPases is dependent on the cellular isoprenoid pool, we treated cells with lovastatin, an inhibitor of HMGR, the enzyme responsible for the rate-limiting step for cholesterol and isoprenoid production. Since lovastatin affects membrane association of sGTPases of both Rho and Rab families, and once we had observed a higher increase in Rab8 membrane content, we have chosen to determine the effect of lovastatin treatment in the CYP46A1-dependent increase in membrane association of this sGTPase. SH-SY5Y cells were transfected with pFLAG-hCYP46A1 or with empty vector and, 24 h afterwards, were treated with vehicle

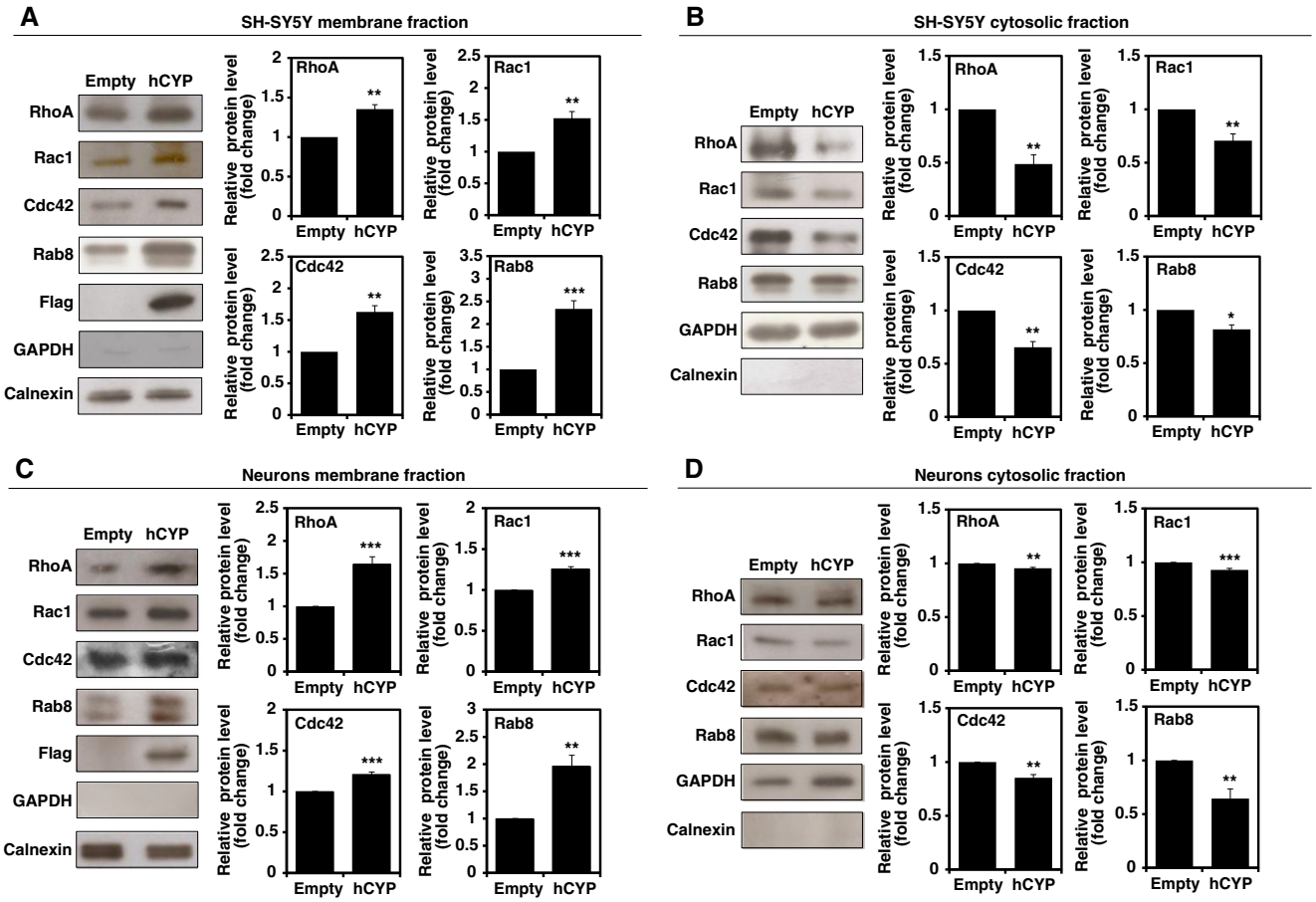

Fig. 2 CYP46A1 overexpression in human SH-SY5Y cells and 26DIV neurons increases sGTPases membrane association. Western blot analysis of sGTPases content in membrane (**a** and **c**) and cytosolic (**b** and **d**) fractions, 48 and 24 h after transfection of SH-SY5Y cells and 26DIV neurons, respectively, with empty pFLAG (*Empty*) or with pFLAG-hCYP46A1 (*hCYP*). *GAPDH* and calnexin levels were used as loading control for cytosolic and membrane fractions, respectively. The blots shown are representative of those obtain in at least three independent experiments. Data is represented as mean values±SEM of at least three experiments and is expressed as fold change relative to control (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

or with 20 μM lovastatin for another 24 h. Not only lovastatin treatment decreased the amount of membrane-associated Rab8 by 60 % in cells transfected with the empty vector but, more interestingly, it completely abolished the increase in Rab8 membrane content induced by CYP46A1 overexpression (two-way ANOVA $p<0.001$, Tukey HSD post hoc test) (Fig. 3).

To further confirm that the increase in CYP46A1 expression correlates with an increase in protein prenylation, we also determined the effect of geranygeranyl transferase inhibition on the CYP46A1-dependent sGTPase membrane association. GGTase-I recognizes protein substrates that have a consensus sequence termed "CAAX box" located at the carboxy terminus, and therefore target proteins of the Rho family. In contrast, proteins of the Rab family undergo two geranylgeranylation reactions and are almost exclusively the substrates for GGTase-II. Nevertheless, it has been shown that some members of the Rab family, such as Rab8, can also be prenylated by GGTase-I [27, 26]. Therefore, we transfected SH-SY5Y cells with pFLAG-hCYP46A1 or with empty vector and after 24 h cells were treated with either vehicle or 50 nM GGTi-2133, a GGTase-I inhibitor, for further 24 h. Finally, the effect on the membrane content of one of the members of the Rho family, RhoA, and on Rab8 was analyzed by immunoblotting. Our results show that GGTi-2133 treatment, as expected, slightly decreases RhoA (15 %), but not Rab8 basal membrane association, while it clearly inhibits the CYP46A1-dependent increase in RhoA (two-way ANOVA $p<0.001$; Tukey HSD post hoc) and Rab8 (two-way ANOVA $p<0.001$; Tukey HSD post hoc test) (Fig. 4).

These results highlight that compounds that either decrease the cellular isoprenoid pool or inhibit geranylgeranylation reactions abolish the increased membrane association of sGTPases upon CYP46A1 overexpression, indicating that the observed differences are due to sGTPase prenylation.

Moreover, we also wanted to evaluate if this increase in sGTPases prenylation was reflected in an increased activation. Therefore, we used a G-LISA kit assay to evaluate RhoA

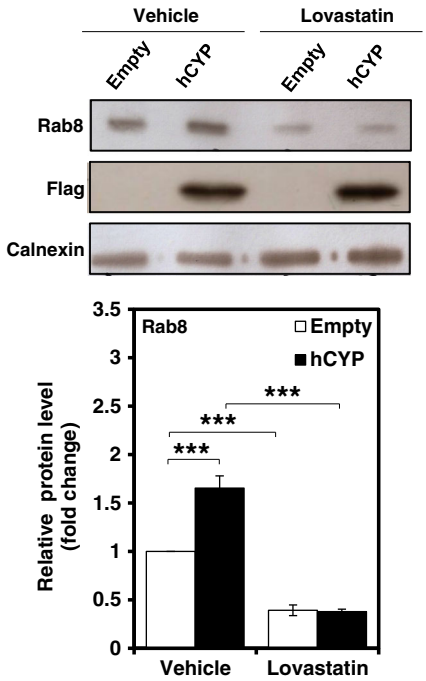

Fig. 3 Lovastatin abolishes the effect of CYP46A1 overexpression on sGTPases membrane association in human SH-SY5Y cells. Western blot analysis of Rab8 content in the membrane fraction isolated from SH-SY5Y cells transfected with empty vector (*Empty*) or pFLAG-hCYP46A1 (*hCYP*) for 24 h and treated for further 24 h with 20 μM lovastatin or vehicle. Calnexin was used as loading control. The blots shown are representative of those obtain in at least three independent experiments. Data is represented as mean values±SEM and is expressed as fold change relative to control (***$p<0.001$)

activity in cell extracts from SH-SY5Y transfected for 24 h with pFLAG-hCYP46A1 or with empty vector and treated with vehicle or 50 nM GGTi-2133 for further 24 h after transfection. Our results show that CYP46A1 significantly increases GTP-bound RhoA by approximately 50 %, which reflects an increase in RhoA activation status. GGTi-2133 treatment completely abrogates the CYP46A1 effect (two-way ANOVA $p<0.05$, Tukey HSD post hoc test) (Fig. 5).

Since CYP46A1 effect seems to be dependent on the supply of isoprenoids, we decided to assess how CYP46A1 overexpression affects the mevalonate pathway, by measuring HMGR activity 48 h after transfection of SH-SY5Y with pFLAG-hCYP46A1 or with empty vector (Fig. 6). The addition of 500 μM pravastatin to non-transfected SH-SY5Y cell homogenates resulted in a 70 % reduction of the basal NADPH consumption, while in the homogenates of cells that were serum starved for 24 h, the NADPH consumption doubled when compared to control (0.47±0.18 nmol NADPH/min/mg protein) (ANOVA $p<0.001$, $F=16.62$, Tukey HSD post hoc test) (Fig. 6a). These data suggested that the NADPH consumption in these homogenates was specifically due to HMGR activity. Interestingly, overexpression of CYP46A1 induces a 2.3-fold significant increase in the rate of NADPH oxidation that is abolished by pravastatin addition, further confirming that this effect is due to an increase in HMGR activity (ANOVA $p<0.05$, $F=5.305$, Tukey HSD post hoc test) (Fig. 6b).

These results are in agreement with the significant increase in the levels of several cholesterol precursors that were previously described in the brains of transgenic mice overexpressing HA-CYP46A1 (C46-HA mice) [16]. To further confirm our observations, we isolated membrane and cytosolic fraction from the brain cortex of C46-HA mice and determined by immunoblotting the content of RhoA, Rac1, Cdc42, and Rab8 in both fractions. We observed an increase in membrane association of RhoA, Rac1, and Rab8 in C46-HA mice relative to wt controls that was similar to what was shown in cultured cells (Fig. 7). The membrane-associated level of RhoA, Rac1, and Rab8 increased by approximately 48, 89, and 18 %, respectively, and this was accompanied by a concomitant decrease in the abundance of sGTPases in the cytosol by 37, 34, and 44 % relative to wt controls. However, in the

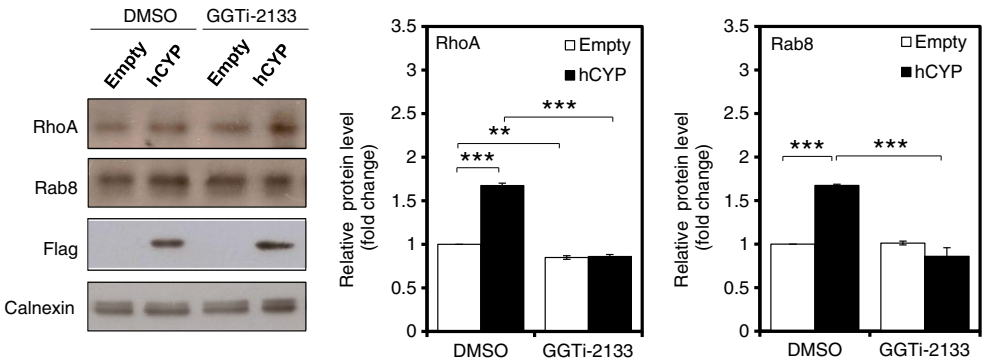

Fig. 4 GGTi-2133 abolishes the effect of CYP46A1 overexpression on sGTPases membrane association in human SH-SY5Y cells. Western blot analysis of RhoA and Rab8 content in membrane fraction in SH-SY5Y cells transfected with empty vector (*Empty*) or pFLAG-hCYP46A1 (*hCYP*) for 24 h and treated for further 24 h with 50 nM GGTi-2133 or vehicle. Calnexin was used as loading control. The blots shown are representative of those obtain in at least three independent experiments. Data is represented as mean values±SEM and is expressed as fold change relative to control (**$p<0.01$, ***$p<0.001$)

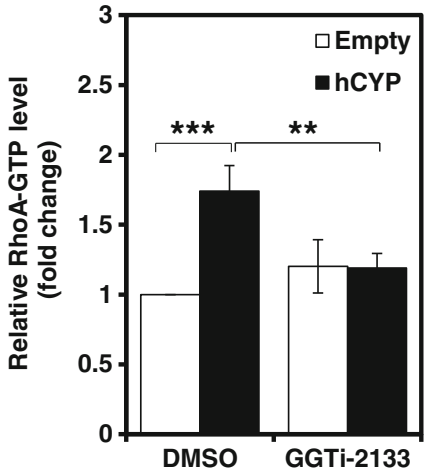

Fig. 5 GGTi-2133 abolishes the effect of CYP46A1 overexpression on RhoA activation status in human SH-SY5Y cells. G-LISA analysis of RhoA-GTP levels in SH-SY5Y cells transfected with empty vector (*Empty*) or pFLAG-hCYP46A1 (*hCYP*) for 24 h and treated for further 24 h with 50 nM GGTi-2133 or vehicle. Data is represented as mean values±SEM of at least three independent experiments and is expressed as fold change relative to control (**$p<0.01$)

case of Cdc42, there was a reduction of approximately 50 % in both membrane and cytosolic fractions, which indicates a reduction of Cdc42 expression in these mice. When the values of membrane-associated Cdc42 are normalized to the total pool of membrane and cytosolic Cdc42, there is no statistical significant change between transgenic and wild-type mice (data not shown).

All together, these results show that CYP46A1 overexpression activates the mevalonate pathway and increases sGTPases prenylation and activation.

24(S)-Hydroxycholesterol, in Contrast to CYP46A1 Overexpression, Reduces RhoA Membrane Association

24OHC is the endogenous product of cholesterol oxidation mediated by CYP46A1, therefore to assess if the observed changes in sGTPases induced by CYP46A1 overexpression were due to an increased production of this oxysterol, 26DIV neurons were incubated with 10 μM 24OHC for 24 h. Since, in vitro, 24OHC activates LXR, in parallel experiments we incubated cells with 10 μM TO901317, an LXR synthetic agonist. Total extracts and membrane fractions were obtained as previously described and the sGTPases content was assessed by Western blot. We observed that both 24OHC and TO901317 had similar effects, but neither recapitulated the overall increase in membrane-associated sGTPases induced by overexpression of CYP46A1. Indeed, although both LXR agonists induced a significant 40 to 50 % increase of Rac1, Rab8, and Cdc42 content in the membrane fraction (Fig. 8), RhoA content in the membrane fraction was reduced by about 35 and 20 % with 24OHC and TO901317, respectively (one-way ANOVA $p<0.001$, $F=18.97$; $p<0.01$, $F=17.37$; $p<0.01$, $F=8.316$; and $p<0.01$, $F=10.78$, corresponding to RhoA, Rac1, Cdc42, and Rab8 analysis, respectively, Dunnet post hoc test) (Fig. 8). These results indicate that CYP46A1 overexpression and 24OHC have similar effects

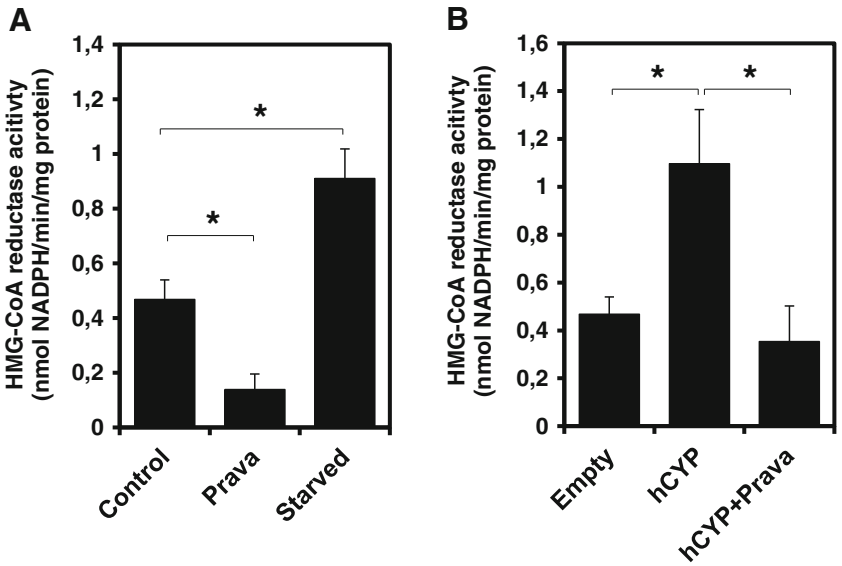

Fig. 6 Overexpression of CYP46A1 in human SH-SY5Y cells increases HMGR activity. HMGR specific activity measured by NADPH consumption was determined in SH-SY5Y cell homogenates. **a** Homogenates prepared from control cells, from 24 h serum-starved cells (*Starved*) or prepared from control cells with addition of 500 μM pravastatin (*Prava*). **b** Homogenates prepared from cells transfected with empty vector (*Empty*) or pFLAGhCYP46A1 (*hCYP*) for 48 h with and without the addition of 500 μM pravastatin (*Prava*). Data is represented as mean values±SEM of at least three independent experiments and is expressed as nanomole of NADPH consumption per minute per milligram of protein (nmol/min/mg) (*$p<0.05$)

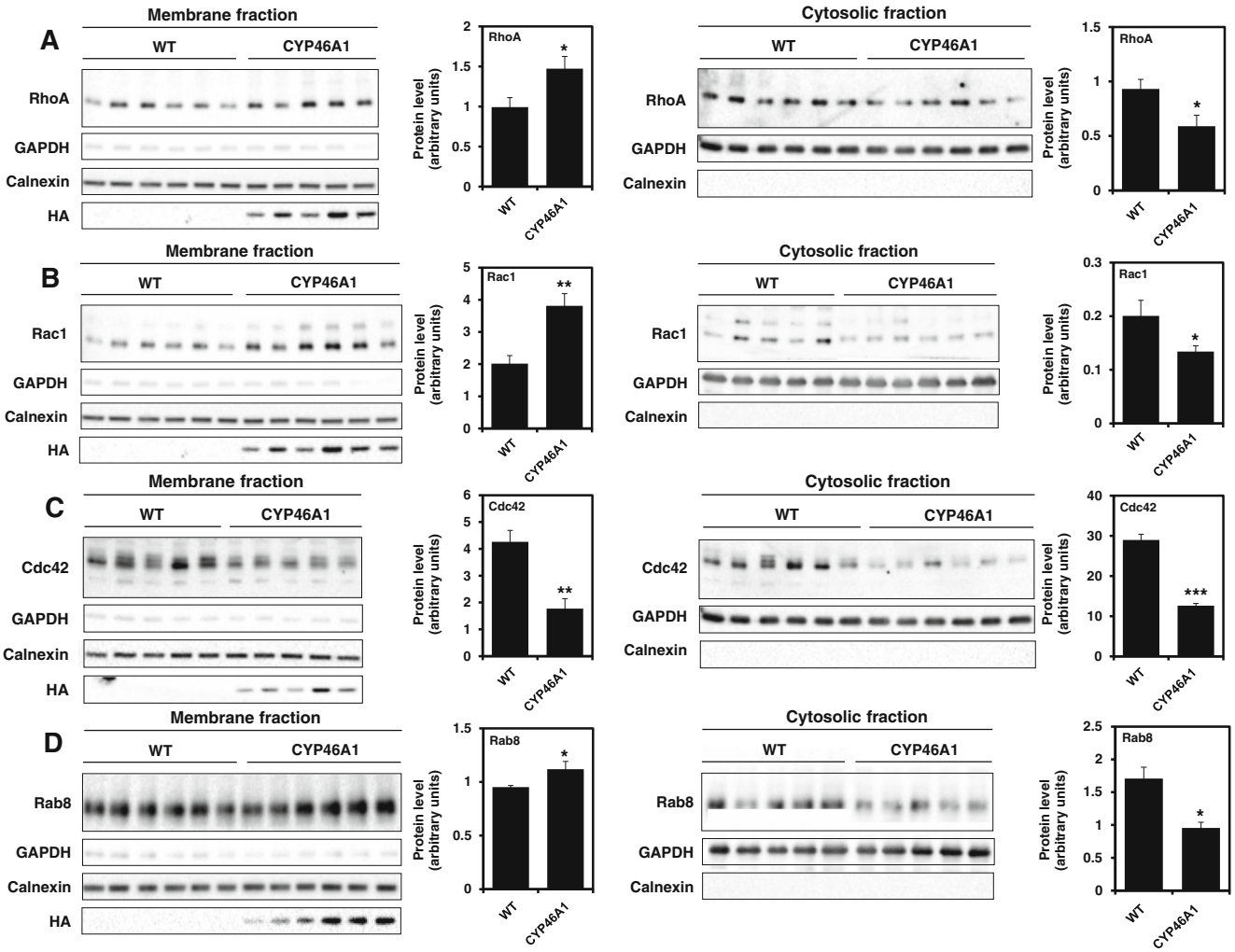

Fig. 7 Brain cortex of C46-HA mice present an increase in sGTPases membrane association. Western blot analysis of sGTPases content in membrane and cytosolic fractions (**a**–**d**) of C46-HA mice brain cortex. *GAPDH* and calnexin levels were used as loading control for cytosolic and membrane fractions, respectively. Data is represented as mean values ±SEM and is expressed as arbitrary units (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

for Rac1, Cdc42, and Rab8 but opposite roles concerning RhoA membrane association.

CYP46A1 Overexpression Inhibits the LXR Pathway as a Consequence of RhoA Activation

As previously mentioned, inactivation of *Cyp46a1* has been associated with a selective reduction of cholesterol synthesis [13], while a significant increase in the levels of several cholesterol precursors was observed in the brain of CYP46A1 transgenic mice [16, 20]. Moreover, herein we have also observed an increase in HMGR activity in CYP46A1 transfected cells. Taken together, these findings suggest a close relationship between synthesis and catabolism of cholesterol in the brain. Therefore, we have determined by qPCR the mRNA levels of key genes involved in cholesterol homeostasis, namely HMGS and HMGR (synthesis), LDLR (uptake), ABCA1, and APOE (efflux) in SH-SY5Y cells transfected with hCYP46A1. Although we had previously observed an increase in HMGR activity, our results show that HMGS and HMGR mRNA levels remain unchanged after CYP46A1 overexpression (Fig. 9a) which is consistent with the gene expression profile reported for C46-HA mice [20]. On the other hand, the mRNA levels of the LXR target genes, ABCA1 and APOE, were significantly down-regulated by approximately 30 and 20 %, respectively. To further confirm the CYP46A1 effect, we also transfected N26DIV neurons with pFLAG-hCYP46A1 and observed similar reductions of ABCA1 and APOE mRNA levels about 15 and 20 %, respectively (Fig. 9b). Furthermore, this decrease is not underlined by a reduction in LXRα and LXRβ expression, since their mRNA levels remain unchanged after CYP46A1 overexpression (data not shown). It was initially hypothesized that an increase in CYP46A1 expression would lead to increased levels of 24OHC and to a general activation of LXR and LXR-targeted genes in the brain. However, the observed

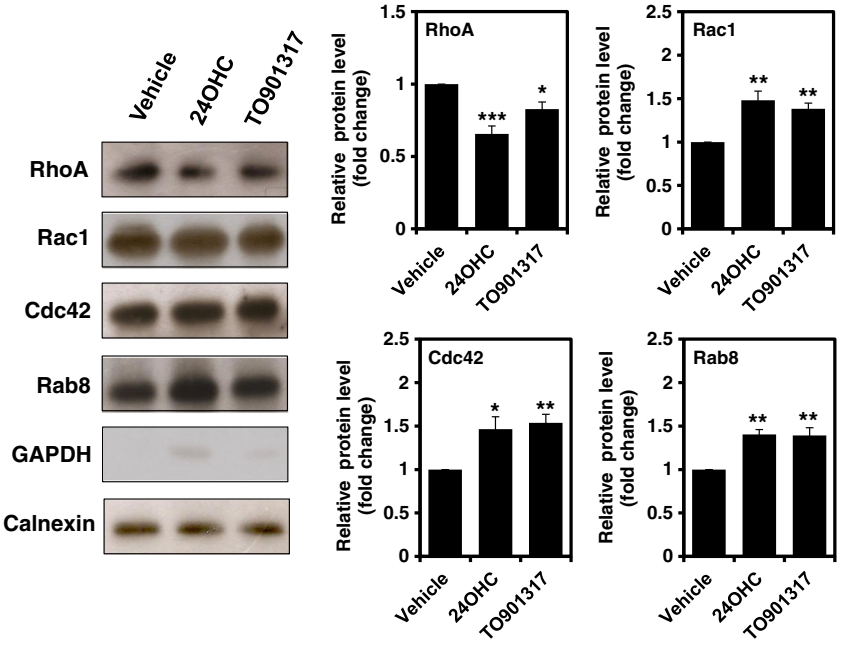

Fig. 8 24OHC and TO901317 affect sGTPases membrane association in 26DIV neurons. Western blot analysis of sGTPases content in the membrane fraction of 26DIV neurons incubated with 10 μM 24OHC or TO901317 for 24 h. Values were normalized to the internal standard calnexin. Data is represented as mean values± SEM of at least three independent experiments and is expressed as fold change relative to control (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

down-regulation of ABCA1 and APOE after CYP46A1 overexpression did not corroborate this hypothesis. Moreover, recent results from Shaffati and collaborators [16], obtained with the transgenic C46-HA mice, also showed that higher

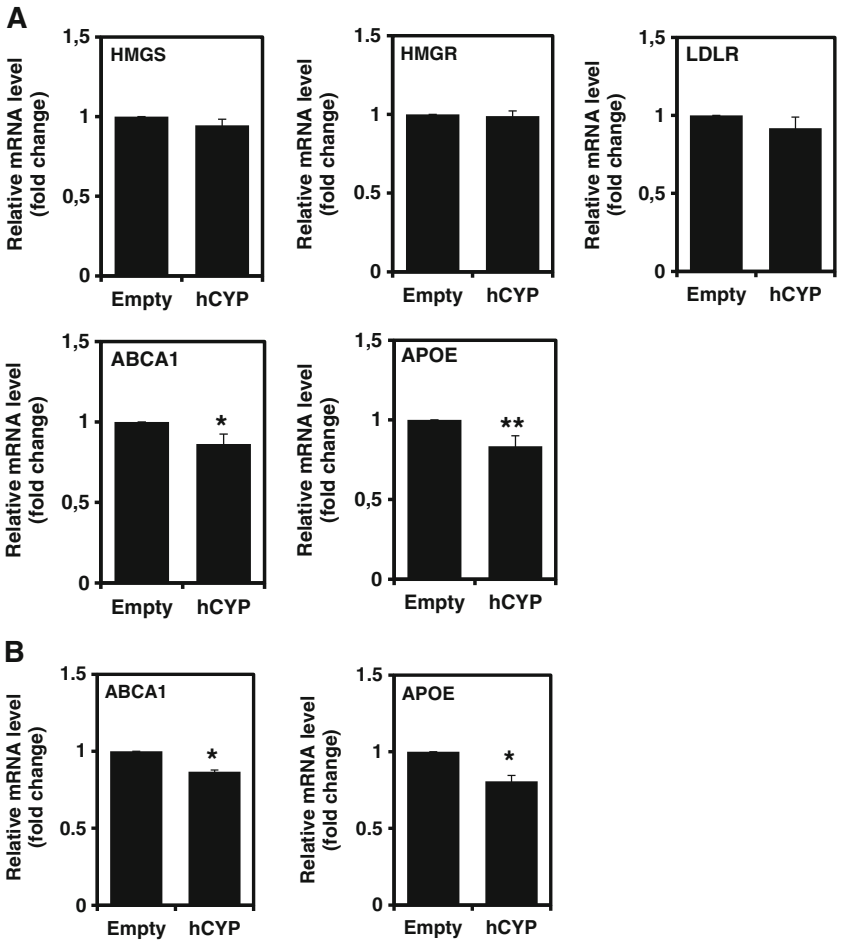

Fig. 9 CYP46A1 overexpression reduces ABCA1 and APOE mRNA levels. **a** qPCR analysis of HMGS, HMGR, LDLR, ABCA1, and APOE mRNA levels in SH-SY5Y cells transfected with empty vector (*Empty*) or pFLAG-hCYP46A1 (*hCYP*) for 48 h. **b** qPCR analysis of ABCA1 and APOE mRNA levels in 26DIV neurons transfected with Empty or hCYP for 24 h. Values were normalized to the internal standard β-actin. Data is represented as mean values±SEM of at least three independent experiments and is expressed as fold change relative to control (*$p<0.05$)

levels 24OHC due to CYP46A1 overexpression were not sufficient to drive LXR activation in vivo.

In order to compare the effect of CYP46A1 overexpression with 24OHC treatment, we have treated 26DIV neurons with 24OHC and determined the mRNA levels of LXR target genes. Our results show that incubation of 10 μM 24OHC increases ABCA1 and APOE mRNA levels by five and twofold, respectively, which is the opposite effect of CYP46A1 overexpression (one-way ANOVA $p<0.001$, $F=8.536$; $p<0.05$, $F=5.577$ for ABCA1 and APOE, respectively, Dunnet post hoc test) (Fig. 10).

Since activation of Rho GTPases has been associated with an inhibition of the LXR pathway [45–49] and we have observed that CYP46A1 overexpression and 24OHC treatment differentially affect RhoA membrane levels, we hypothesize that CYP46A1-dependent decrease in LXR target genes was dependent on the increase in RhoA prenylation. To test our hypothesis, we incubated cells transfected with pFLAG-hCYP46A1 in the presence or absence of GGTi-2133 and observed that inhibition of prenylation abolished the CYP46A1-dependent decrease in ABCA1 and APOE mRNA levels (Fig. 11a). Moreover, we observed that co-transfection of a dominant-negative form of RhoA (pEGFP-RhoA N19) also leads to the loss of the CYP46A1 effect (Fig. 11b). To further confirm that CYP46A1 overexpression affects LXR activation, we performed a reporter gene assay in SH-SY5Y cells co-transfected with the luciferase reporter plasmids LXRE-ABCA1 or LXRE mut-ABCA1 and with empty vector or pFLAG-hCYP46A1 for 48 h (Fig. 12). Upon CYP46A1 overexpression, we observed a 40 % decrease in luciferase activity in cells transfected with the LXRE-ABCA1 luciferase reporter plasmid, an effect that was not observed in cells transfected with the LXRE mut-ABCA1 luciferase reporter plasmid (Fig. 12a). In addition, we have co-transfected the pEGFP-RhoA N19 or the pEGFP empty vector with the luciferase reporter plasmids, and we observed that the CYP46A1-dependent decrease in LXR transcriptional activity was rescued by the inhibition of RhoA activity (Fig. 12b).

These results indicate that overexpression of CYP46A1 can effectively activate sGTPases signaling cascades and have a downstream impact in neuronal cells, namely the down-regulation of the LXR pathway mediated by RhoA.

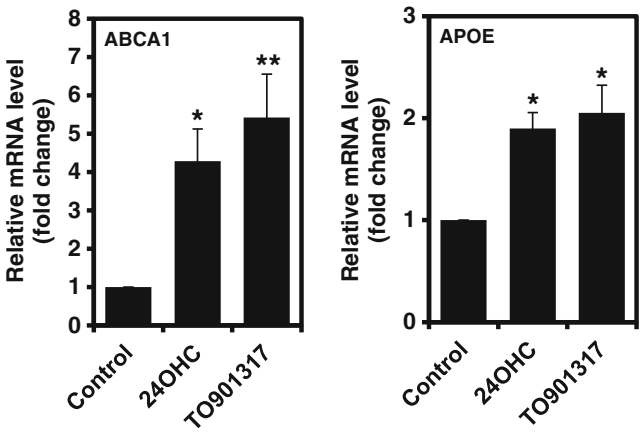

Fig. 10 24OHC and TO901317 increases ABCA1 and APOE mRNA levels in 26DIV neurons. qPCR analysis of ABCA1 and APOE mRNA levels in 26DIV neurons incubated with 10 μM 24OHC or TO901317 for 24 h. Values were normalized to the internal standard β-actin. Data is represented as mean values±SEM of at least three independent experiments and is expressed as fold change relative to control (*$p<0.05$, **$p<0.01$)

Discussion

The present work identifies CYP46A1 as a major player in the regulation of prenylation and activity of sGTPases in neuronal cells. We show that CYP46A1 overexpression induces the activation of the mevalonate pathway, and thereby increases the pool of isoprenoids, as reflected by an increase in prenylation and activation of sGTPases. These results suggest that modulation of CYP46A1 expression can alter the content of prenylated proteins and their activity in neuronal cells, namely that of sGTPases. As mentioned above, these proteins are involved in several CNS pivotal processes ranging from endocytosis to synaptic modulation. Interestingly, *Cyp46a1* null mice have decreased cognitive functions [18, 19] and, in contrast, transgenic mice overexpressing CYP46A1 possess an improvement of cognitive functions [20]. In both cases, these effects were suggested to be due to the inactivation or activation of the mevalonate pathway, respectively. Interestingly, the LTP deficiencies in the knockout mice lacking *Cyp46a1* could not be restored by addition of cholesterol [18] which suggests that cholesterol levels are not directly implicated in cognitive changes elicited by modulation of CYP46A1 expression. In light of these studies and the present results, we put forward the hypothesis that CYP46A1 can influence high-order brain functions such as memory and learning, in part, through modulation of prenylation and activation status of sGTPases. This mechanism may also be part of the explanation for the cognitive improvement in the AD mice model overexpressing CYP46A1 [17]. Indeed, as above-mentioned sGTPases proteins are involved in the control axon growth, guidance and branching, dendritic spine formation and stabilization, growth cone motility and collapse and synapse formation [29–31], and consequently on memory formation, LTP induction and maintenance, and synaptic modulation [31, 33, 34]. We are currently investigation the full impact of CYP46A1-dependent sGTPases activation, namely in terms of neuronal development and function.

Interestingly, in contrast to the effect of CYP46A1 overexpression, incubation with 24OHC decreases membrane association of RhoA, while it increases the membrane abundance of the other sGTPases studied. LXR ligands have been previously

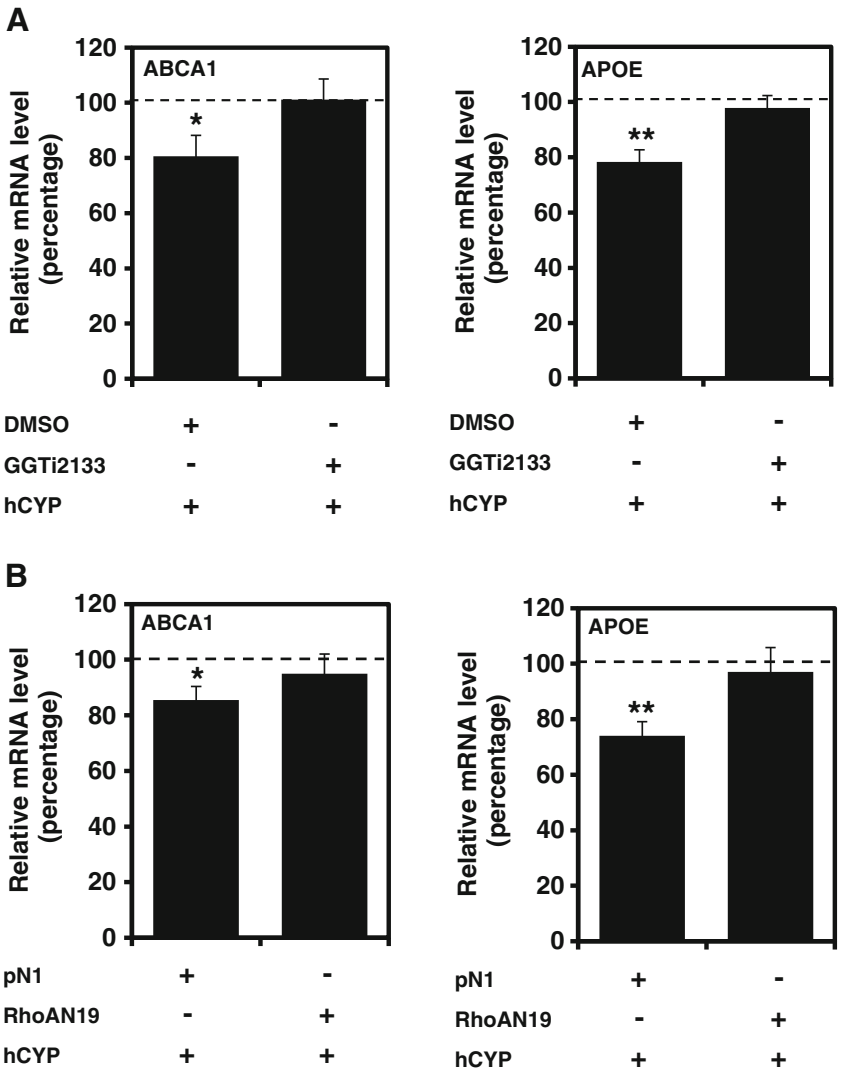

Fig. 11 CYP46A1-dependent inhibition of the LXR pathway is a consequence of sGTPases prenylation. **a** qPCR analysis of ABCA1 and APOE mRNA levels in SH-SY5Y cells transfected with empty vector (*Empty*) or pFLAG-hCYP46A1 (*hCYP*) for 24 h and treated for further 24 h with 50 nM GGTi-2133 or vehicle. **b** qPCR analysis of ABCA1 and APOE mRNA levels in SH-SY5Y cells co-transfected with Empty or hCYP, and with pEGFP empty (*pN1*) or pEGFP-RhoA N19 dominant-negative vector (RhoAN19) for 48 h. Data is represented as mean values±SEM of at least three independent experiments and is expressed as fold change relative to control (*$p<0.05$, **$p<0.01$)

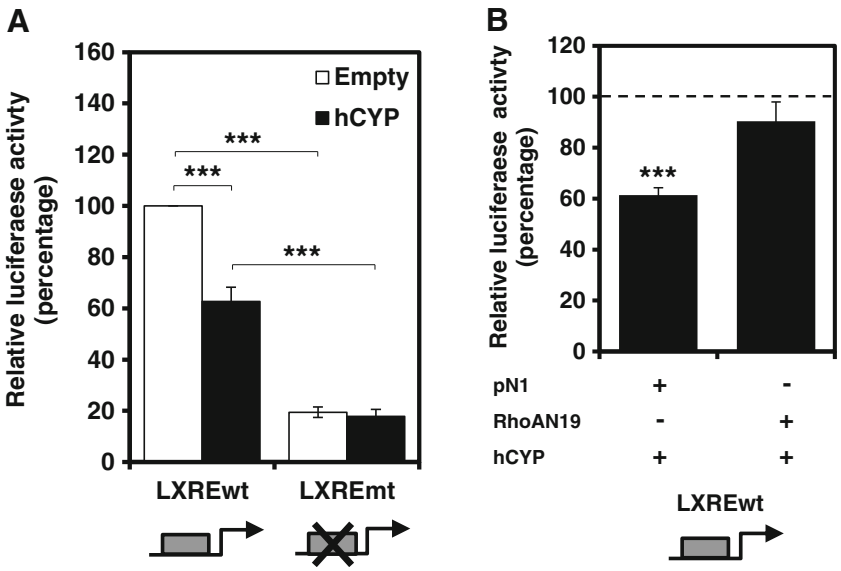

Fig. 12 CYP46A1 overexpression decreases LXR transcriptional activity. **a** LXRE- or LXRE mut-ABCA1 luciferase reporter plasmids were co-transfected with empty vector (*Empty*) or pFLAG-hCYP46A1 (*hCYP*) for 48 h. **b** LXRE-ABCA1 luciferase reporter plasmid was co-transfected with empty vector (*Empty*) or pFLAG-hCYP46A1 (*hCYP*) and with pEGFP empty or pEGFP-RhoA N19 (*RhoAN19*) dominant-negative vector for 48 h. Normalized luciferase activities are expressed as mean values±SEM of duplicates for a minimum of three independent experiments (***$p<0.001$)

shown to have differential effects on the activation of Rho family members, namely by inhibiting RhoA and activating Cdc42 [50, 51]. Moreover, RhoA has been reported to inhibit LXR [45–49], suggesting integration between Rho-dependent functions and LXR activation. Our experiments on the effect of CYP46A1 overexpression on the LXR pathway show that, in contrast to the effect of added 24OHC, increased levels of CYP46A1 reduced the expression of LXR target genes ABCA1 and APOE, which are critical for cholesterol efflux in neurons [52–56]. Interestingly, this decrease was abolished in the presence of a prenyltransferase inhibitor or by the co-transfection of a RhoA dominant-negative mutant. In agreement with the CYP46A1-dependent decrease in ABCA1 and APOE mRNA levels, we observed that the increase in CYP46A1 expression negatively affected the LXR transcriptional activity, and that this decrease was once again rescued by co-transfection of a RhoA dominant-negative mutant. These results strongly indicate that CYP46A1 overexpression can repress the LXR pathway in neurons through the activation of RhoA, in a prenylation-dependent manner. It is tempting to suggest that the present data reflect a global mechanism of cellular response to cholesterol reduction by CYP46A1 in neurons. In order to avoid cholesterol depletion, the cells activate cholesterol synthesis in parallel with a down-regulation of the LXR target genes related to cholesterol efflux (ABCA1 and ApoE). Acute cholesterol depletion by cyclodextrin treatment results in altered membrane properties that can affect synaptic transmission in Purkinje cells and wild-type hippocampal neurons [57, 58]. On the other hand, Sodero and coworkers have suggested that a sudden rise in excitatory neurotransmission activate CYP46A1-dependent mild cholesterol loss mechanisms to protect themselves from neuronal death [59]. Since CYP46A1 activity produces 24OHC, one may speculate that the CYP46A1–sGTPases–LXR axis in neurons is an additional layer of the cell's cholesterol quality control, impairing cholesterol reduction to levels that may affect synaptic transmission. Interestingly, our results may also explain the previous in vivo reports that overexpression of CYP46A1 in mice does not lead to activation of the LXR pathway in spite of the high levels of the efficient LXR agonist 24OHC [16, 17].

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Conflict of Interest The authors declare that they have no conflict of interest.

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