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24S-hydroxycholesterol: cellular effects and variations in brain diseases

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Abstract

The adult brain exhibit a characteristic cholesterol homeostasis, with low synthesis rate and active catabolism. Brain cholesterol turnover is possible thanks to the action of the enzyme Cytochrome P450 46A1 (CYP46A1) or 24-cholesterol hydroxylase, that transforms cholesterol into 24S-hydroxycholesterol (24S-HC). But before crossing the blood-brain barrier (BBB), this oxysterol that is the most abundant in the brain can act locally, affecting the functioning of neurons, astrocytes, oligodendrocytes, and vascular cells. The first part of this review addresses different aspects of 24S-HC production and elimination from the brain. The second part concentrates in the effects of 24S-HC at the cellular level, describing how this oxysterol affects cell viability, amyloid β production, neurotransmission, and transcriptional activity. Finally, the role of 24S-HC in Alzheimer, Huntington and Parkinson diseases, multiple sclerosis and amyotrophic lateral sclerosis, as well as the possibility of using this oxysterol as predictive and/or evolution biomarker in different brain disorders is discussed.

Keywords: 24S-hydroxycholesterol, cholesterol, brain, neurodegeneration, Alzheimer disease, biomarkers of brain disease

Introduction

Cholesterol homeostasis in every tissue is maintained by the delicate mutual regulation between synthesis and catabolism in different cell types. In the mature brain, after myelination has ceased, cholesterol catabolism acquires special relevance to regulate the levels of this lipid, particularly in neurons, due to the general decline of the cholesterol synthesis rate in the brain. The limited synthesis essentially takes place in astrocytes, which have the ability to supply neurons with cholesterol via lipoproptein particles. ATP-binding cassette (ABC) transporters on the astrocyte's plasma membrane are in charge of exporting the synthetized cholesterol to the extracellular milieu. The brain apolipoproteins needed to assamble the lipoprotein particles (ApoE and ApoJ) are also produced and secreted by astrocytes. Ultimately, the lipoprotein particles that carry cholesterol are internalized into neurons after the interaction with surface lipoprotein receptors of the LRP and LDL families (Vitali et al. 2014; Zhang & Liu, 2015) (see Fig. 1).

Cholesterol synthesis in the brain involves the same molecular reactions than in the liver, being the initial precursor Acetyl-CoA. The major regulatory check-point of the cholesterol synthesis is the convertion of 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) to mevalonate by the enzyme 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR), a known target of the statins. The transcription of this enzyme is controlled by sterol regulatory element-binding proteins (SREBPs), transcription factors that can bind an sterol-regulatory element (SRE) in the promoter region of the HMGCR gene. The SREBP family is integrated by three isoforms: SREBP-1a, SREBP-1c, and SREBP-2. Among them SREBP-2 is the most abundant in brain tissue (Horton 2002; DeBose-Boyd and Ye 2018; Camargo et al. 2009; Ferris et al. 2017).

The possibility of using lipophlic statins that cross the BBB to modify brain cholesterol synthesis, in an attempt to compensate for the altered cholesterol homeostasis observed in different diseases, has been initially thought as a plausible intervention (Malfitano et al. 2014). Nontheless, the results obtained in preclinical and clinical trials with statins were not clear enough (Shepardson et al. 2011). At present, the use of low doses of efavirenz to increase the production of 24S-HC via CYP46A1 activation is starting to be regarded as a factible therapy to fight the dysregulated cholesterol homeostasis observed in many pathologies of the brain (Petrov and Pikuleva 2019).

1.1. Brain cholesterol catabolism

The enzymatic specificity for cholesterol catabolism in the entire central nervous system (CNS) is high, with only one enzyme, the CYP46A1, controlling the catabolic rate. Thus, the main cholesterol-derived metabolite is 24S-HC, an oxysterol that is able to cross the BBB and reach the circulation (Björkhem et al. 1997). At the subcellular level, CYP46A1 has been detected at the endoplasmic reticulum (ER) and plasma membrane of hippocampal, cortical and cerebellar neurons (Russell et al. 2009; Sodero et al. 2011, Sodero et al. 2012).

Studies in bovine samples revealed a particular CYP46A1 expression pattern in the retina, with high levels in the neural retina, low levels in the retinal pigment epithelium, and no expression in the ciliary body. Evaluation of the posterior pole of the rat retina showed that CYP46A1 is specifically expressed in neurons but not photoreceptors. In addition, the concentration of 24S-HC is more than 10-fold higher in the neural retina than in the retinal pigment epithelium or the ciliary body (Bretillon et al. 2007). Quantifications of CYP46A1 in the human retina showed an average concentration of 60 fmol/mg of protein in the total membrane fraction, being this concentration 5 times lower than the one measured in the temporal lobe (Liao et al. 2011). The fact that the neural retina specifically expresses CYP46A1 has stimulated researchers to explore whether 24S-HC could be implicated in the onset of age-related macular degeneration and glaucomas (Bretillon et al. 2007).

The brain secretion of 24S-HC is developmentally regulated: the concentration of serum 24S-HC is low in newborn mice, reaches a peak at postnatal days 12-15, and then declines (Lund et al. 1999). In the adulthood, the plasma 24S-HC/cholesterol ratio is about 5 times higher during the first decade than during the sixth decade of life (Lütjohann et al. 1996).

Few is known about the transcriptional regulation of the cholesterol catabolic enzyme CYP46A1. Studies of the promoter region of the human CYP46A1 gene have found that it has a high GC content, a feature of genes with a housekeeping function. One of the stimuli that increases the transcriptional activity of CYP46A1 is oxidative stress (Ohyama et al. 2006; Sodero et al. 2011). The possibility of a substrate-dependent transcriptional regulation of CYP46A1 was analyzed using a sterol-deficient animal model, the 24-dehydrocholesterol reductase (DHCR24) knockout mice, in which most of the cholesterol is replaced by desmosterol. In this scenario with very low levels of cholesterol, the mRNA

levels of CYP46A1 did not change, stressing that cholesterol does not participate in the transcriptional regulation of the catabolic enzyme (Ohyama et al. 2006).

Studies in which the concentrations of 24S-HC was evaluated in the internal jugular vein and the brachial artery in healthy volunteers confirmed a net flux of this oxysterol from the brain into the circulation, congruent with the transformation of approximately 4 mg of cholesterol per day in adults (Lütjohann et al. 1996). Experiments in rats exposed to labeled oxygen were also consistent with a net flux of 24S-HC from the brain into the circulation. The finding that the concentration of 24S-HC is 30- to 1,500-fold higher in the brain than in any other organ, except the adrenals, indicates that most of the 24S-HC present in the circulation is originated in the brain. In addition, there is a high correlation between the 24S-HC concentrations in plasma and cerebro-spinal fluid (CSF) (Lütjohann et al. 1996), what has prompted the exploration of 24S-HC as a plasma biomarker in different neurogical diseases. In humans, all of the 24-HC present in the circulation originates from the brain, and corresponds to the 24(S) stereoisomer. In mice, almost half of the circulating 24-HC is produced in other organs different from brain. Thus, a mixture of the 24(S) and 24(R) isomers is detected in the mouse circulation, being 24S-HC levels approximately 5 times higher than the levels of 24R-HC. As in human brain, the 24S-HC is the most abundant stereoisomer produced in the mouse brain (Björkhem et al. 1997; Saeed et al. 2014).

1.2. Molecular mechanisms of 24S-HC elimination

The molecular mechanisms that allow 24S-HC to cross cell membranes and reach the CSF or blood remained largely unknown. Considering that this metabolite is less hydrophobic than cholesterol, transporters on the plasma membrane of cells that integrate the neurovascular unit should be involved in the elimination process. In vitro studies performed in *Xenopus laevis* oocytes that express the rat organic acid transporter peptide 2 (OATP2) demonstrate that this non-specific transporter can efflux 24S-HC across the cell membrane (Ohtsuki et al. 2007). Experiments made in differentiated SH-SY5Y cells demonstrated that 24S-HC is actively eliminated via the ABCA1 and ABCG1 transporters, that are induced by the intracellular accumulation of this oxysterol. This 24S-HC efflux is stimulated in presence of HDL acceptor particles, but it is not efficient in presence of ApoA-I particles (Matsuda et al. 2013). Similarly, in HEK-293 cells stably expressing human ABCA1

or ABCG1, a good 24S-HC efflux required HDL particles (Matsuda et al. 2013). Considering that the intracellular accumulation of 24S-HC has been shown to be toxic at moderate to high concentrations (above 10 μM; see Section 2.1), the elimination of this oxysterol via ABCA1 or ABCG1 activity would render protection against its intracellular build-up.

1.3. 24S-HC receptors

The metabolite 24S-HC behaves as an agonist of the nuclear liver X receptors (LXRs), influencing the expression of LXR target genes implicated in cholesterol homeostasis and inflammatory responses. The LXR family includes two isoforms: LXRα, that is expressed in liver, kidney, small intestine, spleen, and adipose tissue; and LXRβ, that is abundantly expressed in brain and liver. LXRs form obligate heterodimers with all RXR isoforms (α, β, or γ) that recognize and bind to sequence-specific binding elements within the promoters of target genes to initiate transcription. Normally, LXR:RXR heterodimers associate with DNA sequences irrespective to their ligand binding status. When unbound to ligand, LXRs mediate transcriptional repression by interacting with co-repressors, functionally silencing the expression of target genes (Wagner et al. 2003). In the presence of ligands like oxysterols, the co-repressor complex experiences a conformational change and is replaced by a co-activator complex that triggers transcription. Alternativelly, when LXRs are activated by ligands, these receptors can promote a transrepressive activity, whereby they interact with co-repressor complexes on AP-1 and NFκB target genes, repressing the transcription of pro-inflammatory genes (Saijo et al. 2013; Courtney and Landreth 2016).

An induction of the transporters ABCA1 and ABCG1 by 24S-HC has been demonstrated in differentiated SH-SY5Y cells (Matsuda et al. 2013) and conditionally-immortalized choroid plexus epithelial cells (Fujiyoshi et al. 2007). In a similar way, the treatment of brain pericytes in culture with 24S-HC increases the expression of ABCA1, and this correlates with cholesterol transfer to Apo-E, ApoA-I, and HDL particles, while inhibition of ABCA1 decreases this cholesterol efflux (Saint-Pol et al. 2012). It has also been reported that 24S-HC behaves as a high affinity ligand for the retinoic acid receptor-related orphan receptors α and γ (RORα and RORγ; Ki ≅ 25 nM). The cholesterol metabolite acts as inverse agonist, suppressing the constitutive transcriptional activity of these receptors in co-transfection assays performed in HEK-293 cells. In addition, 24S-HC suppresses the expression of

two RORα target genes, BMAL1 and REV-ERBα, decreasing the recruitment of the coactivator Src-2 to the promoter of these genes in HepG2 cells (Wang et al. 2010).

1.4. CYP46A1 knockout and overexpression

In CYP46A1 knockout mice, despite a great reduction in the abundance of brain 24S-HC, no other cholesterol metabolite was found to quantitatively replace this oxysterol. In addition, the levels of brain cholesterol in CYP46A1 knockout mice are similar to those of wild-type mice (Kotti et al. 2006; Meljon et al. 2014). However, the levels of the cholesterol precursor desmosterol, and its parallel metabolite 24S,25-epoxycholesterol, are lower in CYP46A1 knockout mice (Meljon et al. 2014). These studies suggest a reduction in the cholesterol synthesis rate with normal levels of this lipid in the brain of CYP46A1 knockout mice. A geranyl-geranyol deficiency due to a drop in the cholesterol synthesis rate has been associated to learning and memory as well as long-term potentiation (LTP) deficits in CYP46A1 knockout mice (Kotti et al. 2006; Kotti et al. 2008). Transcriptional and proteomic studies in mice contributed to identify additional effects induced by the knockout of this catabolic enzyme. First, there was a compensatory up-regulation of the intracellular cholesterol storage pathways in CYP46A1 knockout brains. Second, the decreased cholesterol synthesis in CYP46A1 knockout brains was due to inhibition of SREBP-2 processing and retention of the SREBP-2/SCAP complex in the endoplasmic reticulum. Third, some of the LXR target genes (i.e. ABCA1) were paradoxically upregulated in CYP46A1 knockout brains, possibly due to a reduced activation of the small GTPases Rab8 and Cdc42. Fourth, the phosphorylation of 146 proteins was altered in CYP46A1 knockout brains, including the microtubule-associated protein (MAP) and neurofilament proteins (NEF family), along with proteins involved in synaptic transmission (SLC, SHANK and BSN). Fifth, the extent of protein ubiquitination was increased in CYP46A1 knockout brains, and the affected proteins were related to ubiquitination (UBE2N), cognition (STX1B and ATP1A2), cytoskeleton function (TUBA1A and YWHAZ), and energy production (ATP1A2 and ALDOA) (Mast et al. 2017).

The effects of CYP46A1 overesperession were analyzed using a transgenic mouse model in which human CYP46A1 was expressed under the control of the β-actin gene promoter (Shafaati et al. 2011). Both male and female overexpressing mice exibited elevated levels of brain, plasma, biliary

and fecal 24S-HC. Surprisingly, gene expression profiling revealed that the elevated production of 24S-HC did not result in the activation of LXR target genes in the brain, in spite of the fact that 24S-HC has been found to be a potent LXR agonist in astrocyte cultures (Abildayeva et al. 2006). Thus, this finding in CYP46A1 overexpressing mice strongly suggests that 24S-HC, even at brain concentrations 2-times above the physiological range, is not a good activator of the LXRs in vivo.

1.5. Pharmacological modulation of 24S-HC production

Initial research on the crystal structure of CYP46A1 predicted that ligands other than sterols can bind this enzyme (Mast et al. 2008). Later, the antifungal drug voriconazole was found to bind CYP46A1 in vitro and inhibit cholesterol hydroxylation with a Ki of ~11 nM (Shafaati et al. 2010). In addition, seven-week old mice treated with voriconazole for 5 days (60 or 75 mg/kg/day i.p.) exhibited high levels of voriconazole and reduced levels of 24S-HC in the brain. The levels of squalene, lathosterol, and HMGCR mRNA were lower in the brain of voriconazole-treated compared to vehicle-treated animals, suggesting a reduction in cholesterol synthesis, produced by a diminished transformation of cholesterol into 24S-HC. Remarkably, one of the side-effects of voriconazole is visual disturbances (Huber and Stahlmann 2012), in agreement with the high expression of CYP46A1 in the neural retina (Bretillon et al. 2007).

Another drug that has been shown to modulate CYP46A1 activity in vitro and in vivo is the antiviral efavirenz, which increases the production of 24S-HC (Mast et al. 2014; Anderson et al. 2016). Importantly, recent in vitro studies demonstrate that the primary metabolites of efavirenz are stronger and better activators of CYP46A1 than efavirenz (Mast et al. 2020). It remains to be determined if CYP46A1 activity can be potentiated by these metabolites in vivo.

2. Cellular effects of 24S-HC

2.1. Cell viability

The main brain-derived cholesterol metabolite 24S-HC has been shown to induce cell death, either by necrosis or apoptosis, depending on the cell type and tested concentration. So far, two important molecular players have been identified in association to the toxic effects of this oxysterol: Ca2+/calmodulin protein kinase II (CaMKII) (Kimura et al. 2018) and receptor-interacting serine/threonine-protein kinase 1 (RIPK1) (Vo et al. 2015). Although the activation of both CaMKII

and RIPK1 leads to cell damage and posterior cell death, the existing literature supports that these two enzymes are not regulated each other.

In non-differentiated human neuroblastoma SH-SY5Y cells, 24S-HC induces RIPK1-dependent morphological changes, compatible with necrosis (Yamanaka et al. 2011). The exposure of these cells to physiological concentrations of 24S-HC drastically reduces cell viability in 48 hours (65% and 95%, at 10 and 50 μM, respectively), and this toxic effect is preceded by an intracellular Ca2+ peak, 30 hours after treatment initiation (Kölsch et al. 1999). Conversely, in differentiated SH-SY5Y cells, 24S-HC induces apoptosis, evidenced by DNA-fragmentation, caspase-3 activation, reduction of the mitochondrial membrane potential, and generation of free radicals. This toxicity in differentiated SH-SY5Y cells is partially prevented by physiological concentrations of vitamin E (50-100 μM) (Kölsch et al. 2001). A comparison between SH-SY5Y and T-lymphoma Jurkat cells, allowed to conclude that the lack of caspase 8 expression in SH-SY5Y is responsible for the absence of apoptosis in response to 24S-HC.

In Jurkat cells, 24S-HC induces the formation of lipid droplets, intracellular organelles made up of a lipid esters/triglyecerides core covered by a monolayer of phospholipids, cholesterol, and associated proteins. It is accepted that the main function of the lipid droplets is the storage of lipid esters, although fatty acid and steroid synthesis have been detected in these dynamic organelles (Farmer et al. 2020; Fujimoto and Parton 2011). The appearance of lipid droplets in Jurkat cells requires the activity of the acetyl-CoA acetyltransferase (ACAT1) and is associated to cell death. The 24S-HC also induces lipid droplets formation in SH-SY5Y cells through an ACAT1-dependent process, and the disappearance of lipid droplets by inhibition or knock-down of this enzyme recovers cell viability (Yamanaka et al. 2014).

The treatment of SH-SY5Y cells with sub-lethal concentrations of 24S-HC induces a reduction in the amount of cell death triggered by the subsequent administration of 7-ketocholesterol, in both undifferentiated and retinoic acid-differentiated SH-SY5Y cells. The co-treatment 24S-HC/retinoic acid enhances the protective response of 24S-HC, and the administration of 24S-HC induces the expression of the LXR target genes ABCA1 and ABCG1. These results suggest that 24S-HC at sub-lethal concentrations promotes an adaptive transcriptional activation of the LXR signaling pathway, thereby protecting cells from toxicity (Okabe et al. 2013). At low concentrations, 24S-HC decreases

the staurosporine-mediated induction of caspase-3 and caspase-7, although at high concentrations this oxysterol exacerbates the toxic effects of staurosporine (Emanuelsson and Norlin 2012). In addition, 24S-HC induces apoptosis via caspase-3 activation in SH-SY5Y cells differentiated with retinoic acid, in which caspase-8 expression is induced. In retinoic acid-treated cells, ROS generation is not induced by 24S-HC. Instead, 24S-HC estrification followed by lipid droplets formation due to ACAT1 activity are key events in the 24S-HC-induced cell death, and the number of lipid droplets is not sensitive to antioxidant treatments (Nakazawa et al. 2017).

The toxicity of 24S-HC was also evaluated in murine oligodendrocytes in culture. This oxysterol induces a decay in cell proliferation, modification of mitochondrial activity, overproduction of ROS, caspase 3 activation, PARP degradation, reduced expression of Bcl-2, and condensation and/or fragmentation of the nuclei, all changes that highlight increased oxidative stress and apoptosis. Moreover, 24S-HC promotes the conversion of microtubule-associated protein light chain 3 (LC3-I) to LC3-II, which is a hallmark of autophagy. It was proposed that 24S-HC leads to cell damage through "oxiapoptophagy", a process that is attenuated by antioxidant vitamins (Nury et al. 2015). In addition, 24S-HC modifies the lipid content and polarization of the cytoplasmic plasma membrane, leading to higher intracellular concentrations of K+. The blockage of voltage-dependent K+ channels exacerbates 24S-HC-induced cell dysfunction, inhibiting cell growth, augmenting the mitochondrial depolarization and cytoplasmic membrane damage, and increasing the percentage of sub-G1 cells (Bezine et al. 2017). Furthermore, 24S-HC increases the expression levels of Kv3.1b channels in 158N murine oligodendrocytes and BV-2 microglial cells (Bezine et al. 2018). Altogether, these results show that an abnormal regulation of the intracellular concentration of K+ is implicated in the cytotoxicity induced by 24S-HC.

Considering that most of the described experiments where the 24S-HC toxicity was evaluated were performed in proliferative neuroblastama cell lines, additional studies in different types of neuronal cultures, including human stem cell derived-neurons, are necessary to understand the molecular actions of 24S-HC in a more neuronal post-mitotic context.

2.2. Amyloid β production

It has been demonstrated that the cholesterol metabolite 24S-HC can affect the enzymatic proccesing of the transmembrane amyloid precursor protein (APP). This protein is sequentially cleaved, in the first place by the β-secretase, and then by the γ-secretase complex, to produce the hydrophobic amyloid β peptide (Aβ). The inheritance of mutations in APP or presenilins (PSN1 and PSN2), that constitute the catalytic core of the γ-secretase complex, cause increased generation of Aβ and familiar Alzheimer disease (AD).

In SH-SY5Y cells in culture, 24S-HC increases the endogenous α-secretase activity as well as the α-secretase to β-secretase activity ratio (Famer et al. 2007), indicating a shift towards the non-amyloidogenic processing of APP, what could be beneficial in the context of AD. In another work performed in the same cell line, 24S-HC increased the secretetion of APPα, but did not affect the production of Aβ42, suggesting that APP processing via the non-amyloidogenic pathway is favored by this oxysterol (Prasanthi et al. 2009). Contrasting with these results, in differentiated SK-N-BE human neuroblastoma cells, 24S-HC induced a net synthesis of Aβ42 by up-regulating the expression of APP and β-secretase as well as the β-secretase activity. Interestingly, N-acetyl-cysteine pre-treatment fully prevented the enhancement of amyloidogenesis induced by this oxysterol (Gamba et al. 2014).

In addition to the capacity of modulating APP processing, it has been demonstrated that 24S-HC can influence the binding of the hydrophobic Aβ peptide to different surface proteins. Particularly, 24S-HC enhances the binding of Aβ to human differentiated neuronal cell lines (SK-N-BE and NT-2), by up-regulation of the CD36 and β1-integrin membrane receptors. At a low concentration of 1 μM, 24S-HC markedly potentiates the pro-apoptotic and pro-necrogenic effects of Aβ42 on these cells, through the NADPH oxidase-dependent generation of ROS, and the consequent impairment of the redox equilibrium. The antioxidants quercetin and genistein prevent the pro-oxidant effects of 24S-HC and the potentiation of Aβ-induced necrosis or apoptosis. These results have led to the proposal that the presence of 24S-HC in the close vicinity of the amyloid plaques could enhance the adhesion of large amounts of Aβ to the plasma membrane of neurons, amplifying the neurotoxic action of this peptide (Gamba et al. 2011).

The oxysterol 24S-HC reduces Aβ production and increases ER-resident immature APP levels in SH-SY5Y and CHO cells stably expressing human APP. Treatment with 1-10 μM of 24S-HC

(equivalent to the concentrations detected in human brain homogenates) diminishes Aβ production without affecting the secretase activities. The APP budding via COPII-coated vesicles is strongly diminished in 24S-HC-treated cells. This oxysterol induces the expression of the ER chaperone glucose-regulated protein 78 (GRP78), through unfolded protein response pathways, and enhances the formation of the APP/GRP78 complex (Urano et al. 2013). Altogehter, these results evidence that 24S-HC can down-regulate the APP trafficking via enhancement of the APP/GRP78 complex formation in the ER, resulting in the suppression of Aβ production.

In addition to influencing Aβ physiology, it was shown that 24S-HC contributes to prevent the neurotoxic accumulation of the hyperphosphorylated tau protein, that takes place in the cytoskeleton of diseased AD-neurons. This protective effect involves the modulation of the protein deacetylase SIRT-1 (Testa et al. 2018).

2.3. Neurotransmission

The main excitatory neurotransmitter in the mammalian brain is the aminoacid glutamate that is able to interact with specific ionotropic or metabotropic receptors disseminated in different synapses. Between them, the NMDA receptor (NMDAr) subtype, whose stimulation is associated to increased neuronal influx of Ca2+, is critically involved in learning and memory processes as well as toxicity mediated by its over-activation (excitotoxicity).

It has been demonstrated that 24S-HC behaves as a positive allosteric modulator of the NMDAr. At sub-μM concentrations, this oxysterol potentiates the NMDAR-mediated excitatory postsynaptic currents (EPSCs) in rat hippocampal neurons, but fails to affect currents mediated by AMPA or GABA-A receptors. In addition, in hippocampal slices, 24S-HC potentiates the ability of subthreshold stimuli to induce long-term potentiation (LTP), and reverses LTP deficits induced by blockage of the NMDAr with ketamine. Interestintly, synthetic derivatives of 24S-HC, which potently enhance NMDAR-mediated EPSCs and LTP, restore behavioral and cognitive deficits in rodents treated with NMDAr channel blockers (Paul et al, 2013). Complementary studies in hippocampal slices of CYP46A1 knockout mice, where endogenous 24S-HC levels were greatly reduced, showed a reduction in the NMDAr tone, diminishing the spiking events driven by the NMDAr. The administration of SGE-301, a 24S-HC analog, had comparable potentiating effects on NMDAr EPSCs in both wild-type and CYP46A1 knockout slices, suggesting that endogenous 24S-HC does not

saturate its NMDAR modulatory site. Knockout slices did not differ from wild-type slices in either spontaneous neurotransmission or neuronal intrinsic excitability, and showed an LTP indistinguishable from wild-type slices. However, knockout slices exhibited higher resistance to persistent NMDAR-dependent depression of synaptic transmission induced by oxygen-glucose deprivation, an effect that could be rescued by SGE-301 (Sun et al. 2016). It was suggested that a loss of NMDAr tone does not elicit compensatory changes in excitability, but protects neurotransmission against NMDAr-mediated dysfunction. In addition, it was demonstrated that 24S-HC (1 μM) enhances tonic, but not evoked, NMDAr-mediated currents in the dentate gyrus granule cells of the hippocampus. In contrast, in CYP46A1 knockout mice, 24S-HC slightly decreases the tonic NMDA-mediated currents in the granule cells. Additionally, 24S-HC has no effect on tonic NMDAr-mediated currents in the parvalbumin interneurons of the hippocampal dentate gyrus (Wei et al. 2019).

There is also evidence that 24S-HC can affect the availability of precursors needed for the synthesis of neurotransmitters. For example, in rat hippocampal synaptosomes treated with methyl-β-cyclodextrin to reduce the amount of membrane cholesterol, 24S-HC (50 μM) diminished the high affinity choline transport, required for acetylcholine synthesis. This effect was counteracted by aggregated Aβ42 peptides that can interact with this oxysterol (Kristofiková et al. 2008). Data from computational simulation indicate that the hydrophobic core of Aβ binds the hydrophobic region of the 24S-HC molecule, but with no formation of stable hydrogen bonds (Kristofiková et al. 2012).

The effect of 24S-HC on the recycling of synaptic vesicles has been specifically explored at the mouse neuromuscular junction. The treatment with this oxysterol increases the end-plate potential (EPP) amplitude in response to a single stimulus and diminishes the EPP amplitude rundown during high frequency activity, but had no influence on miniature EPP amplitude or frequency. Comparison of electrophysiological and optical data revealed an increase in the vesicle recycling rate. The impact of 24S-HC can be abolished or potentiated by stimulation or inhibition of the NMDAr, respectively. Moreover, 24S-HC mimicks the effect of an endothelial NO synthase (NOS) inhibitor, suppressing the generation of NO during high frecuency stimulation, while glutamate produces the opposite effect. Inhibitors of the endothelial NOS (L-NAME and cavtratin), but not the neuronal NOS (TRIM), a scavenger of extracellular NO and a protein kinase G blocker, all have stimulatory effects similar to those of 24S-HC, on exocytosis induced by high frequency stimulation, and completely mask the 24S-

HC effects. Altogether, these data demonstrate that 24S-HC enhances synaptic vesicle cycling due to an attenuation of retrograde NO signaling that involves endothelial NOS activity. In this regard, 24S-HC counteracts the effects of NMDAr stimulation at mouse neuromuscular junctions (Kasimov et al. 2017). In rat PC12 cells, 24S-HC stimulates the vesicle fusion to the plasma membrane promoting exocytosis (Ma et al. 2010). This increased exocytosis may result in high levels of synaptic glutamate, leading to excitotoxicity, particularly in brain areas undergoing inflammation, where the concentrations of cholesterol oxidation products are high.

The effects of 24S-HC (0.4 μM) on neurotransmission were also investigated in the diaphragm of transgenic mice carrying a mutant SOD1 (SODG93A). In this experimental model of amyotrophic lateral sclerosis (ALS), 24S-HC suppressed the spontaneous neurotransmitter exoxytosis and release upon high-frequency stimulation. The latter was paralelled by a decay in both the vesicle recycling rate and the activity-dependent enhancement of NO synthesis. The inhibition of NO synthase diminished synaptic vesicle exocytosis during high-frequency stimulation, and completely abolished the 24S-HC effect. This oxysterol also increased the synaptic membrane lipid ordering, and the effects of 24S-HC on lipid rafts and NO production could be blocked by lipid raft-disrupting agents, like methyl-β-cyclodextrin and sphingomyelinase (Mukhutdinova et al. 2018). These experiments in transgenic SODG93A mice demonstrate that 24S-HC is able to suppress the exocytotic release of neurotransmitter in response to intense synaptic activity, via a NO/lipid raft-dependent mechanism.

2.4. Transcriptional changes

The expression of many enzymes in the cholesterol/isoprenoid and fatty acid synthesis pathways are regulated by SREBPs, transcription factors that are both transcriptionally and post-transcriptionally regulated. Proteomic data indicates that 24S-HC (10 μM for 24 hours) down-regulates cholesterol synthesis in rat cortical neurons in culture via SREBP-2, most likely, in a post-transcriptional manner. In contrast, the enzymes implicated in the synthesis of fatty acids are not down-regulated in cortical neurons treated with 24S-HC, while Apo-E is up-regulated. Specifically, 24S-HC down-regulates the expression of members of the cholesterol/isoprenoid synthesis pathways, including 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1, diphosphomevalonate decarboxylase, isopentenyl-diphosphate delta isomerase, farnesyl-diphosphate synthase, and the sterol synthesis

enzymes, farnesyl-diphosphate farnesyltransferase 1 and methyl-sterol monooxygenase. Therefore, if cholesterol accumulates intracellularly in neurons, the formed 24S-HC promotes a dual effect: down-regulation of key enzymes involved in cholesterol synthesis, through SREBP-2, and up-regulation of Apo-E synthesis, through LXR activation (Wang et al. 2008).

DNA arrays and western blot analysis in primary co-cultures of human neurons and glial cells revealed that the treatment with 10 μM 24S-HC for 24 hours increases the expression levels of inducible cyclooxygenase 2 (COX-2), phospholipase A2 (PLA2), heat-shock protein 70 (HSP70), and APP, not affecting the expression of the glial fibrillar acidic protein (GFAP) or β-III-tubulin genes (Alexandrov et al. 2005). The observed increase in the expression of the pro-inflamatory genes COX-2 and PLA2 is reverted by simvastatin (Alexandrov et al. 2005), a lipophilic statin that can penetrate into the brain and produce pleiotropic effects on neurons and astrocytes (Sodero and Barrantes 2020).

3. 24S-HC in animal models of brain disease

3.1. Alzheimer disease

The role of the 24S-HC-producing enzyme CYP46A1 has been investigated in different mouse models of AD, using downregulation and overexpression strategies, to elucidate if the modification of this enzyme's activity is able to influence the course of the experimental disease. For example, Djelti and collegues (Djelti et al. 2015) used an adeno-associated virus (AAV) vector encoding a short-hairpin RNA directed against the mouse CYP46A1 mRNA, to reduce the expression of the CYP46A1 gene in hippocampal neurons of wild-type mice. This manipulation increased the cholesterol concentration in neurons, and produced cognitive deficits and hippocampal atrophy due to apoptotic neuronal death. Prior to neurodegeneration, recruitment of APP into lipid rafts, paralleled by increased production of Aβ-carboxi-terminal fragments and Aβ, were observed, together with abnormal phosphorylation of tau and endoplasmic reticulum stress.

In the APP23 mouse model of AD that overexpresses human mutant APP, the inhibition of CYP46A1 using the same viral vector increased the concentration of Aβ peptides, and neuronal death was more extensive than in control mice (Djelti et al. 2015). The injection of an AAV vector encoding CYP46A1 in the cortex and hippocampus of APP23 mice at 3 months of age, before the onset of amyloid deposits, markedly reduced the levels of Aβ peptides, the number of amyloid deposits, and

the levels of trimeric Aβ oligomers at 12 months of age. These aninals showed a prior improvement of spatial memory at 6 months, before the onset of amyloid deposition (Hudry E, 2010). Overexpression of CYP46A1 by injection of the same AAV vector in the cortex and hippocampus of APP/PS1 mice, after the onset of amyloid deposits, reduced the number of amyloid plaques 3 months after the injection (Hudry E, 2010). Thus, neuronal overexpression of CYP46A1 either before or after the onset of amyloid plaques reduces Aβ pathology in two different mouse models of AD.

Experiments aimed at enhancing the activity of CYP46A1 in 5xFAD mice, a model of rapid amyloidogenesis, were performed using a low dose of efavirenz (0.1 mg/kg/day). This drug was administered from 1 to 9 months of age, and mice were evaluated at specific time points. At 1 month of age, brain cholesterol homeostasis was already disturbed in 5xFAD mice; nevertheless, efavirenz was still able to activate the cerebral cholesterol turnover mediated by CYP46A1 during the first 4 months of administration. This treatment extension also reduced amyloid burden and microglia activation in the cortex and subiculum of 5xFAD mice as well as protein levels of APP and the expression of several inflammation-related genes. However, mouse short- and long-term spatial memories remained impaired. Additional 4 months of efavirenz administration (a total of 8 months) improved long-term spatial memory, further decreased Aβ content in 5xFAD brains, and diminished the mortality rate among male mice (Mast et al. 2017). Complementary experiments using the same low dose of efavirenz (0.1 mg/kg/day), but applying another administration protocol, were performed in 5xFAD mice. The treatment started at 3 months of age, after amyloid plague appearance, and continued for additional 6 months. Under these conditions, efavirenz produced CYP46A1 activation in the brain, enhanced the cholesterol turnover, improved spatial learning, reduced microglia activation, but increased astrocyte reactivity. The levels of the soluble and insoluble Aβ40 and Aβ42 peptides remained unchanged, while the number and area of the dense core amyloid plaques were slightly decreased. Changes in the levels of several synaptic proteins (Munc13-1, PSD-95, gephyrin, synaptophysin, synapsin-1, and calbindin-D28k) were measured, suggesting that CYP46A1 activation by efavirenz affects synaptic function (Petrov et al. 2019). Remarkably, 5xFAD mice treated with efavirenz showed altered expression and phosphorylation of 66 genes and 77 proteins, respectively. Whole transcriptome sequencing (RNA-seq) data suggest that efavirenz affects the synapse, plasmin-depended amyloid clearance, inflammation and microglia phenotype, oxidative stress and cellular

hypoxia, autophagy and ubiquitin-proteasome systems, and apoptosis. It was speculated that these effects could be caused, at least in part, via changes in Ca2+, small GTPases, and catenin signaling. The authors proposed that CYP46A1-dependent lipid raft rearrangement and subsequent decrease in the phosphorylation of certain proteins could be central for the efavirenz-mediated improvements observed in AD mice (Petrov et al. 2019).

In 3xAD mice, deletion of the acyl-CoA cholesterol acyltransferase 1 (ACAT1) produced a reduction in full-length APPswe as well as its proteolytic fragments, and reverted cognitive deficits. At 4 months of age, ACAT1 ablation increased the levels of 24S-HC, reduced the expression of HMGCR, and decreased the cholesterol synthesis rate in mouse brains. In agreement with these findings in vivo, ACAT1 deletion in cultured hippocampal neurons increased 24S-HC production, and 24S-HC administration caused a rapid decline in human APPswe and HMGCR protein amounts (Bryleva et al. 2010).

In mice that express human tau without developing tau pathology (hTau mice), i.c.v. injections of 24S-HC can completely prevent the hyperphosphorylation of tau induced by Aβ42 monomers (Testa et al. 2018). These results highlight the importance of preventing the loss of brain 24S-HC in order to counteract neurodegeneration.

Degeneration of cholinergic neurons expressing choline acetyltransferase (ChAT) in the basal nucleus of Meynert (nBM) and a decay in cholinergic activity correlate with cognitive decline in AD. Studies in nBM rat slices showed that 24S-HC administration reduces the decay in the number of ChAT-positive neurons caused by nerve growth factor deprivation (Ullrich et al. 2010).

The feeding of wild-type mice with a high-cholesterol diet for 5 months induces cognitive deficits, which are accompanied by increased levels of CYP46A1 mRNA and 24S-HC, as well as BACE1 and Aβ proteins, in the hippocampus and cortex. The addition of polyphenols from oriental plum to the high-cholesterol diet reverted all these changes (Kuo et al. 2015).

3.2. Huntington disease (HD)

This autosomal dominant disorder is characterized by progressive neurodegeneration caused by the expansion of the CAG repeat on the amino-terminal region of the huntingtin protein. The

neuropathological hallmark of HD is the severe atrophy of the striatum, which produces involuntary movements (chorea) and cognitive dysfunction.

It has been demonstrated that CYP46A1 expresssion is reduced in the striatum of R6/2 mice, a transgenic model of HD that expresses the first exon of the human huntingtin gene containing 150 CAG repeats. CYP46A1 mRNA levels were decreased in the striatum and cortex but not in the hippocampus of transgenic animals at 6 and 12 weeks of age. Decreased CYP46A1 protein levels were also observed in these brain areas at 12 weeks of age (Boussicault et al. 2016). In a comparative study using immortalized wild-type (STHdhQ7) and mutant (STHdhQ111) striatal neuronal progenitor cell lines expressing huntingtin, with either 7 or 111 glutamines, respectively, the STHdhQ111 striatal cell line exhibited a reduction of CYP46A1 mRNA and protein levels when compared to STHdhQ7 striatal cells. Cholesterol and 24S-HC measurements in the striatum of R6/2 mice (12 weeks-old) showed a 1.5-fold increase of cholesterol in the striatum, therefore reproducing observations made in the caudate of HD patients (Del Toro et al. 2010). Filipin labelling of cholesterol showed a marked staining of plasma membrane in both wild-type and R6/2 striatal cells, with absence of cholesterol accumulation in internal organelles of R6/2 mice. Despite decreased expression of CYP46A1, 24S-HC concentration was not altered in the striatum of R6/2 mice (Boussicault et al. 2016). Interestingly, overexpression of CYP46A1 in primary striatal neuronal cultures expressing mutated huntingtin with 82 glutamine repeats (mHtt82Q) and in the striatum of YAC128 mice demonstrated that CYP46A1 is protective against excitotoxicity (Boussicault et al. 2018).

The YAC128 mouse model of HD shows a reduction of 24S-HC levels in the brain and plasma at 10 months of age, consistent with a diminution of several components of the cholesterogenic pathway. On the contrary, mice overexpressing the wild-type protein with 18 CAG reapeats (YAC18 mice) show the opposite phenotype with elevated activity of the cholesterol synthetic pathway. In addition, at 2 months of age the brains of YAC128 mice exibit unchanged 24S-HC levels combined with reduced concentrations of lanosterol and lathosterol, implying that the biosynthesis defect occurs early in life, followed by a progressive decrease in total cholesterol, associated to decreased production of 24S-HC due to reduced substrate concentrations (Valenza et al. 2007). Finally, in the transgenic R6/1 mice, 24S-HC showed reduced levels in the striatum at 28 weeks of age. The

cholesterol precursors lathosterol and lanosterol were also reduced in the cortex and striatum by 6 weeks of age, prior to the onset of motor dysfunction and cognitive abnormalities (Kreilaus et al. 2015).

3.3. Hypoxia-ischemia (HI)

Experiments made in C57BL/6 pups at postnatal day 9 showed that a HI procedure induces a transient cholesterol loss, 6 hours after the insult. Importantly, the cholesterol catabolic enzyme CYP46A1 is up-regulated 6 and 24 hours after the insult, with a concomitant increase of 24S-HC in the ipsilateral cortex and serum. In addition, after 6 and 24 hours of HI, the levels of 24S-HC in serum correlated with those in the brain, as well as with necrotic and apoptotic cell death evaluated by the expression of spectrin breakdown products and cleaved caspase-3 (Lu et al. 2018). Altogether, these pre-clinical results evidence that serum 24S-HC could be used as a severity biomarker in hypoxic-ischemic encephalopathies.

3.4. Traumatic brain injury (TBI)

Under physiological conditions, most brain CYP46A1 is expressed in neurons, with very low levels detected in glia. Interestingly, the levels of CYP46A1, measured by immunoblotting, increase in the rat parietal cortex 7 days afer TBI. Cell type specific analysis 3 days after TBI showed an abnormal increase of CYP46A1 in microglial cells of the cortex. ApoE and ABCA1, two known LXR-regulated genes, increased 7 days post-TBI, indicating that increased LXR activity coincided with higher CYP46A1 levels. In vitro, the activation of primary rat microglia with LPS increased the levels of CYP46A1. It was proposed that the increased microglial CYP46A1 activity is needed to remove damaged cell membranes after TBI, by conversion of cholesterol into 24S-HC, and activation of LXR-regulated genes by this oxysterol (Cartagena et al. 2008). In cultured neuroblastoma cells, 24S-HC increased the levels of SREBP-1 but did not change its processing, consistent with the role of 24S-HC as an LXR agonist. In contrast, 24S-HC decreased the levels of the LXR-independent SREBP-2 and its cleavage product. In addition, 24S-HC decreased the mRNA levels of the cholesterol synthesis genes HMGCR, squalene synthase, and farnesyl pyrophosphate synthase but did not alter the mRNA levels of acetyl CoA carboxylase or fatty acid synthase, involved in fatty acid

synthesis. After TBI in mice, as after 24S-HC treatment in vitro, SREBP-1 mRNA levels were increased while SREBP-2 mRNA levels were decreased. Also similar to the in vitro findings with 24S-HC, HMGCR and squalene synthase mRNA levels were decreased. Fatty acid synthase mRNA levels were not altered but acetyl CoA carboxylase mRNA levels were decreased (Cartagena et al. 2010). Therefore, TBI alters the transcription of cholesterol synthesis genes, upregulating CYP46A1 activity, but does not modify fatty acid synthesis.

3.5. Eye diseases

Studies on the role of 24S-HC in the etiology of glaucoma were performed in Sprague-Dawley rats submitted to laser photocoagulation of the trabecular meshwork, limbus and episcleral veins in one eye, to elevate the intraocular pressure (Fourgeux et al. 2012). Both eyes and plasma were analyzed at increasing time points after the photocoagulation procedure, which caused a sustained elevation of the intra-ocular pressure in the lesioned eye that lasted 21 days. Increased concentrations of MCP-1 and ICAM-1 were detected in plasma 3 days after the lesion. Glial activation was observed bilaterally at all the evaluated time-points, at lower levels in contralateral eyes than in the injured eyes. Remarkably, in photocoagulated retinas, CYP46A1 expression showed a transient increase at day 3, while plasma and retinal 24S-HC peaked at days 14 and 30, respectively. These data demostrate that CYP46A1 induction is an early event in response to retinal stress, and reinforce the idea that CYP46A1 has a constitutive role in maintaining neuronal cholesterol balance.

In another work, the impact of CYP46A1 inhibition by voriconazole was tested in the rat retina. Five days of voriconazole administration (60 mg/kg/day i.p.) decreased 24S-HC levels in the retina and impaired its functioning. Curiously, CYP46A1 and GFAP expression was higher in the retina of voriconazole-treated compared to control rats, and ICAM-1 and MCP-1 increased in both the retina and vitreous body of treated animals. These data highlight a cross-talk between retinal ganglion cells and glial cells in the retina, and suggest that the drop in neuronal 24S-HC production is detected by glial cells, which are consequently activated (Fourgeux et al. 2014).

Glaucoma is a progressive and irreversible blinding neuropathy that is characterized by the loss of retinal ganglion cells. These neuronal cells permanently interact with Müller glial cells, the most numerous glial cells in the retina in charge of sustaining cholesterol homeostasis. These glial cells are

activated in retinal gliosis, and overproduction of 24S-HC is associated to glaucoma. Experiments performed in raft and non-raft fractions purified from cultured Müller glial cells demonstrated that the addition of 10 μM 24S-HC led to an alteration in the composition of the lipid rafts, with enrichment of cholesterol, sphingomyelin, saturated fatty acids, and ganglioside GM3, and the proteins caveolin-1, flotillin-1, connexin-30, and connexin-43 (Gambert et al. 2017).

The retina was also investigated in CYP46A1 knockout mice that have an average 1.8-fold increase in the amount of retinal cholesterol (Saadane et al. 2019). The retina of these transgenic animals showed venous beading and tortuosity, microglia/macrophage activation, and high vascular permeability, all hallmarks of the diabetic retinopathy. The expression of LXRα and LXRβ was increased in both the whole CYP46A1 knockout retina and retinal microglia/macrophages in culture. In microglia/macrophages, the LXR-transactivated genes ABCA1, ABCG1, Apo-D, Apo-E, Mylip, and Arg2 were up-regulated. Similarly, there was an up-regulation of the LXR-transrepressed genes Ccl2, Ptgs2, Cxcl1, Il1b, Il6, Nos2, and TNFα. CYP46A1 detection was positive in retinal endothelial cells, and the expression of this enzyme increased in the proinflammatory environment. Retinal CYP46A1 knockout phospho-proteome revealed altered phosphorylation of 30 proteins, including the tight-junction protein zonula occludens 1 (ZO-1) and aquaporin 4 (AQP4). Collectively, these data highlight the significance of CYP46A1 for retinal cholesterol homeostasis, and suggest that pharmacologic activation of CYP46A1 may have potential to fight dyslipidemia-induced retinal damage.

3.6. Renin-angiotensin system (RAS)

It has been shown that a high cholesterol diet up-regulates the RAS in mouse brain, increasing the levels of angiotensin converting enzyme (ACE) and angiotensinogen (AGT), and promoting the phosphorylation of JAK/STAT. These effects were also observed in primary cultures of neurons and astrocytes exposed to 1 μM 24S-HC for 24 hours, and were partially mediated by LXR stimulation. In contrast, brain RAS activity was decreased in CYP27A1 knockout mice, which have a reduced 27-HC production. Moreover, normocholesterolemic patients with elevated levels of 27-HC due to a CYP7B1 mutation, exhibited markers of RAS activation in their CSF. These results demonstrate that both oxysterols, 24S-HC and 27-HC, are modulators of the brain RAS. Considering that the levels of

cholesterol and 27-HC correlate in the circulation, and 27-HC can cross the BBB, it was proposed that this oxysterol could represent a link between hypercholesterolemia, hypertension, and brain disease (Mateos et al. 2011). In addition, in rat primary neurons, the levels of ACE2 and Mas receptor (MasR) increased after treatment with a physiological concentration of 24S-HC (1 μM) for 24 hours (Mateos et al. 2012). Interestingly, increased ACE activity and AGT levels have been reported in the CSF of patients with mild cognitive impairment (MCI) and AD (Mateos et al. 2011b). Thus, because cholesterol homeostais in the brain is efficiently protected of high circulating cholesterol levels by the BBB, the possibility of cholesterol metabolites permeating the barrier and affecting the brain may explain the found association between hypercholesterolemia/hypertension and neurodegeneration.

4. 24S-HC in brain diseases

In 1972, Smith and colleagues (Smith et al. 1972) comfirmed the detection of 24-HC in distinct areas of the human brain, including the cortex, subcortical white matter, midbrain, pons, and cerebellum. Quantifying by gass chromatography (GC), they found that the average amount of 24-HC was ~60 μg/g of dried tissue in all the analyzed regions except the cerebellum, where the 24-HC amount was about 3 times lower. In the following decades, multiple methodological sofistications were developed to measure very low amounts of the stereoisomer 24S-HC in plasma and CSF (Griffiths et al. 2016), where the concentrations are very low. Currently, the use of high performace liquid chromatography (HPLC) or GC, in combination with mass spectrometry (MS), assure a reliable quantification of the 24S-HC levels. In human plasma, the concentrations of this oxysterol fluctuate between 10 and 80 ng/mL, while in the CSF they vary between 0.5 and 3 ng/mL, depending on the technical protocol aplied before the oxysterol measurement. The 24S-HC can be determined either as the free molecule or the total molecule after alkaline hydrolysis of 24S-HC esters. In addition, the detection can be performed with or without derivatization of the sample (Honda et al. 2009; Bandaru el al. 2014; Sidhu et al. 2015; Sugimoto et al. 2015; Novakova et al. 2015).

4.1. Alzheimer disease and other dementias

Non-inherited late onset AD is the most prevalent form of dementia, characterized by a gradual decline in memory and other cognitive and executive functions, and the progressive development of

behavioral disorders. The reported changes in the 24S-HC levels in plasma and CSF of patients with different types of dementia are sumarized in Table 1.

Lütjohann and coworkers (Lütjohann et al. 2000) reported higher 24S-HC concentrations in the plasma of AD and non-AD demented patients, compared to healthy individuals and patients with depression. In addition, plasma 24S-HC levels positively correlated with the severity of the dementia. Whether the plasma 24S-HC concentrations are dependent on the severity of AD and ApoE genotype was also investigated (Papassotiropoulos et al. 2000). AD severity and ApoE ε4 allele inheritance were independently associated to reduced plasma 24S-HC/cholesterol ratios. A decrease in this ratio, without changes in the cholesterol levels, was observed in severely affected AD patients, most likely reflecting a loss of brain CYP46A1 due to neuronal death. In the same direction, Bretillon and colleagues (Bretillon et al. 2000) found that subjects with brain death occurring 6-10 hours before the collection of the plasma samples had markedly reduced circulating levels of 24S-HC. Patients with advanced AD and cerebral inflammatory diseases had slightly lower levels of this oxysterol in plasma. Additionally, the brains of AD patients showed a marked difference in the distribution of CYP46A1, with a reduction in the positive staining of this enzyme in neurons, and a higher staining in glial cells (Bogdanovic et al. 2001).

Elevated concentrations of 24S-HC were found in the CSF of AD patients, suggesting an increased turnover of brain cholesterol due to neurodegeneration. A gene-dosage effect of the ApoE ε4 allele on the CSF 24S-HC concentrations was observed in AD patients. The elevation of CSF 24S-HC may occur early in the disease process, since patients with MCI also showed increased CSF concentrations of this oxysterol (Papassotiropoulos et al. 2002).

In another interesting study (Schönknecht et al. 2002), the CSF concentrations of 24S-HC were measured in AD patients and healthy controls with normal plasma cholesterol levels (150-230 mg/dL), to exclude the potential influence of circulating cholesterol on CSF 24S-HC levels. AD patients showed higher 24S-HC levels in CSF, but not in plasma. These results evidence that 24S-HC increases in the CSF of AD patients, in a peripheric cholesterol-independent manner. In addition, it was shown that 24S-HC decreases while the liver-derived metabolite 27-HC increases in brain samples of AD patients (Heverin et al. 2004). The 27-HC/24S-HC ratio increased in the frontal cortex, occipital cortex, basal ganglia, and pons of AD patients, with absence of changes in the protein

levels of CYP46A1 and CYP27A1 in these brain areas. A high correlation was observed between the levels of 24S-HC and lathosterol in the frontal cortex of AD patients but not in controls, indicative of a close coupling between cholesterol synthesis and catabolism in AD brains. It was proposed that the high levels of 27-HC in the brain derive from an increased influx of this oxysterol due to BBB damage, being the low 24S-HC levels a direct consequence of the neurodegenerative process. Kölsch and coworkers (Kölsch et al. 2004) demonstrated that the cholesterol corrected concentrations of plasma 24S-HC and 27-HC are lower in patients with AD, MCI, and vascular dementia (VD), compared to non-demented subjects and depressed patients.

In a comparison between pacients with late onset AD, VD, cognitive impairment and cognitively normal controls (Zuliani et al. 2011), plasma 24S-HC levels were higher in late onset AD and lower in VD. A positive correlation between the 24S-HC/cholesterol ratio and the C reactive protein (CRP) concentration was found in the plasma of late onset AD patients and cognitively impaired individuals, being the correlation stronger in AD patients. In multivariate regression tests that included age, gender, albumin ratio, and ApoE ε4 inheritance, cholesterol, 24S-HC and 27-HC concentrations in the CSF could independently predict both the sAPPα and sAPPβ levels in the CSF (Popp J, 2012). All these associations remained significant when the analysis was separately performed in AD patients. Li and collegues (Li et al. 2018) reported that plasma 24S-HC and Aβ levels are higher in AD patients than controls, suggesting that 24S-HC can be used as a biomarker in the diagnosis of AD.

In another study, Leoni and collegues (Leoni et al. 2013) compared individuals with subjective cognitive impairment (SCI), MCI patients, MCI patients with later progression into AD (MCI-AD), and AD patients, analyzing different biomamarkers in the CSF. In the four studied diseases, the concentrations of 24S-HC, cholesterol, and ApoE were higher than in controls. While the Aβ42 concentration was reduced both in MCI-AD and AD, the levels of total-tau and phospho-tau only increased in AD patients. The fraction of the population with levels of 24S-HC considered to be pathological (> 3 μg/L) was 8% in controls, 34% in SCI, 37% in MCI, 80% in MCI-AD, and 42% in AD. In addition, in a population of old healthy subjects (75-99 years-old) that was analyzed in parallel, there were no individuals with 24S-HC concentrations above the cut-off level of 3 μg/L, whereas the fraction of subjects with high levels of at least one of the common AD biomarkers (total-tau, phopho-tau or Aβ42) was 40%. Based on these percentages, this study argues that the diagnostic

power of 24S-HC in CSF is similar to or lower than that of total-tau, phospho-tau, and Aβ42 in the diagnosis of established AD.

Although at present a decrease in the CSF concentration of Aβ42 is believed to be the most specific and early sign of AD, the increase of the 24S-HC levels in the CSF seems to be more sensitive to detect the prodromal phase of the neurodegenerative process. In addition, the above discussed reports on 24S-HC levels in AD support the existence of a breaking point at which the concentration of this oxysterol in plasma starts to diminish most likely due a massive loss of CYP46A1 expressing neurons at the end phase of the disease. Only three studies (see Table 1) detected higher plasma 24S-HC concentrations in the AD population, suggesting that most of these individuals were undergoing the early phase of the disease with brain cholesterol accumulation.

4.2. Huntington disease

It was reported that plasma cholesterol levels are similar between controls, pre-HD subjects and HD patients. In contrast, the plasma 24S-HC concentrations were lower in HD patients than in controls at all disease stages (Leoni et al. 2008). Interestingly, the plasma 24S-HC concentration of the pre-HD subjects was similar to those of controls. The pre-HD cohort of subjects presented heterogeneous 24S-HC levels, with subjects closer to the predicted development of motor signs having lower 24S-HC levels than those far from the onset. These data indicate that the concentration of this oxysterol is reduced in the plasma of HD patients at any disease stage, and can discriminate pre-manifest subjects from patients with overt motor disease. Nontheless, 24S-HC levels fail to predict further disease progression in patients with manifest HD. In addition, 24S-HC levels parallel the large decrease in caudate volumes observed in gene-positive subjects transiting from pre-manifest to overt HD, therefore reflecting a critical phase characterized by neuronal loss. The plasma 24S-HC concentrations of three groups of gene-expanded individuals (low, medium and high), classified according to a score that considered CAG repeats and age at study initiation, along with a group of non gene-expanded controls were also evaluated (Leoni et al. 2013). A decrease in the levels of 24S-HC as the HD groups progressed from low to high gene expansions was identified. In addition, the lower concentrations of this oxysterol in plasma were paralleled by reduced striatal volumes. Thus, 24S-HC seems to be a proper biomarker to monitor the progression of HD.

4.3. Parkinson disease (PD)

In a complete study in which both the plasma and CSF concentrations of 24S-HC and 27-HC were evaluated in PD patients with different disease duration, all the patients exibited normal circulating levels of these oxysterols. Nevertheless, the CSF analysis showed that 10% of the PD patients had 24S-HC concentrations above the cut-off level, and there was a correlation between the CSF levels of 24S-HC and the disease duration. The CSF concentrations of 27-HC were also found to be above the cut-off level in 10% of the patients, indicating a possible defect in the BBB function, but there was no correlation between the CSF levels of 27-HC and the duration of the disease (Björkhem et al. 2013). These data suggests that the CSF concentration of 24S-HC may represent a valuable biomarker to monitor the progression of PD.

4.4. Multiple sclerosis (MS)

The most common inflammatory demyelinating disease of the CNS it is believed to be an immune disorder that develops in genetically susceptible subjects. Although the exact etiology remains unknown, there is strong evidence suggesting that the primary target of the immune system are the myelin-producing oligodendrocytes. Axonal damage occurs in addition to demyelination and this could be the cause of the later permanent disability.

In MS patients transiting the third and fourth decades of life, the 24S-HC concentrations in the CSF are reduced, probably reflecting neuronal loss, and an inverse corelation between disability and plasma 24S-HC was detected (Leoni et al. 2002). Serum 24S-HC levels are low in primary progressive and older relapse-remitting MS patients (Teunissen et al. 2003). Van de Kraats and collegues (van de Kraats et al. 2014) reported decreased concentrations of 24S-HC and 27-HC in the serum of MS patients. These changes in cholesterol homeostasis would be related to neurodegeneration. In another study, the concentrations of several oxysterols and apolipoproteins were measured at baseline and over five years in MS patients (Fellows Maxwell et al. 2019). The levels of plasma 24S-HC increased in progressive MS patients, and changes in ApoC-II and Apo-E were positively associated with all oxysterol levels. Increases in 24S-HC and Apo-B were observed in relapse-remitting patients who converted to secondary progressive MS at follow-up and in secondary

progressive MS patients, compared with relapse-remitting patients. In another study, the levels of 24S-HC, 27-HC and 7α-HC were reported to be reduced in MS patients compared to healthy controls, and LDL-cholesterol concentrations were associated with higher levels of 24S-HC, 25-HC, 7-KC, and 7α-HC (Mukhopadhyay et al. 2017).

Interestinlgy, natalizumab, a drug that reduces inflammation and neurodegeneration, disminishes the concentrations of 24S-HC and 27-HC in CSF, and the 24S-HC concentration in serum of relapse-remitting MS patients. These reductions in the levels of 24S-HC and 27-HC in the CSF indicate slow-down of the neurodegeneration and better integrity of the BBB, respectively (Novakova et al. 2015).

4.5. Amyothropic lateral sclerosis (ALS)

This neurodegenerative disorder is characterized by the initial denervation of the skeletal muscle and the subsequent death of motor neurons. A characteristic dying-back pattern suggests a preponderant role for neuromuscular junction dysfunction in ALS.

The oxysterol 24S-HC is esterified by the enzyme LCAT in the CSF, and the levels of 24S-HC esters in CSF exibit good correlation with the levels of 24S-HC esters in plasma. The CSF concentrations of 24S-HC esters are lower in ALS patients than healthy subjects. Similarly, the plasma concentrations of 24S-HC esters are lower in patients than in controls. The LCAT amount in the CSF, measured by immunoblotting, is about 4-fold higher in ALS patients than in controls (La Marca et al. 2016). Because oxidative stress was found to reduce the LCAT activity in vitro, and 24S-HC was an effective stimulator of the LCAT secretion in astrocytoma cells in culture, it was hypothesized that the reduced 24S-HC esterification observed in ALS patients would be a consequence of oxidative stress accumualation. Thus, enhanced astrocytic LCAT secretion may represent an adaptive response to the increase of non-esterified 24S-HC, aimed to avoid the accumulation of this neurotoxic compound. Additionally, the low degree of 24S-HC esterification in CSF and plasma may reflect a lower activity of LCAT during neurodegeneration.

4.6. Age-related macular degeneration (AMD)

Macrophage aging is a pathogenic feature in AMD, a leading cause of blindness in older adults. Despite their frequent self-renewal, macrophages from old mice exhibit numerous signs of aging,

such as impaired oxidative respiration. Transcriptomic profiling of aged murine macrophages revealed dysregulation of diverse cellular pathways, especially those involved in cholesterol homeostasis, that leaded to altered oxysterol levels. Importantly, increased plasma 24S-HC levels showed specific association with AMD, demostrating that this oxysterol can discriminate AMD from physiological aging (Lin et al. 2018).

4.7. Cerebrovascular disease

Elevated 24S-HC concentrations in plasma are associated with brain infarcts on prior magnetic resonance imaging. In addition, subjects with high plasma 24S-HC and a greater 24S-HC/27-HC ratio are prone to incident cognitive impairment over 8 years of follow-up (Hughes et al. 2012). Thus, the increased 24S-HC levels suggest increased cholesterol catabolism occurring in the brains of individuals with cerebrovascular disease, prior to the onset of cognitive deterioration.

4.8. Autism spectrum disorders (ASD)

Children with ASD exibit elevated levels of 24S-HC in plasma, and the oxysterol concentration inversely correlate with age. Multivariate analysis established that a high plasma level of 24S-HC is an independent risk factor for ASD (Grayaa et al. 2018). These findings suggests that 24S-HC can be used as a diagnostic tool for ASD.

4.9. Suicides

Post-mortem analysis of human prefrontal cortex tissue shows an increase of 24S-HC in suicide cases, suggesting a higher turnover of cholesterol in this brain region (Freemantle et al. 2013). This increase in cortical 24S-HC, in combination with LXR activation, could explain the observed reduction in both brain and circulating cholesterol concentrations in suicide cases.

5. 24S-HC in systemic diseases

5.1. Hyperlipidemia

Familial combined hyperlipidemia (FCHL), the most common inherited disorder of lipid metabolism, is characterized by high levels of cholesterol precursors due to hepatic overproduction of

cholesterol. All serum oxysterol levels are high in individuals with FCHL in comparison to healthy controls, with about 60% of the subjects presenting 24S-HC values above the 95th percentile for this oxysterol in the control population. FCHL subjects with oxysterol overproduction present a diminution of the carotid intima media thickness, that suggests reduced atherosclerosis (Baila-Rueda et al. 2014). These findings indicate that high serum oxysterol levels could be good markers of FCHL, and exert a protective mechanism against cholesterol accumulation.

The effect of the lipophilic statin simvastatin was evaluated in hypercholesterolemic patients, either after 6 or 24 weeks of treatment (Locatelli et al. 2002). Simvastatin reduced the total plasma cholesterol levels and the plasma lathosterol/cholesterol ratio (an index of hepatic cholesterol synthesis) in a similar extent with both treatment extensions. Surprisingly, plasma 24S-HC levels and its ratio to cholesterol also disminished after these treatments, highlighting the impact of simvastatin in the brain, where it reduces cholesterol synthesis and 24S-HC production.

Oxysterols were quantified in plasma and arteries with atherosclerotic plaques from patients with severe peripheral artery disease (PAD) as well as arteries free of atherosclerotic plaques of control subjects. The levels of 24S-HC and 27-HC were 5- and 20-fold higher, respectively, in the arterial tissue of PAD individuals than in those of controls. The concentration of CRP in plasma correlated with plasma 24S-HC and 27-HC levels, and with tissue 24S-HC and 27-HC amounts. The authors conluded that the arterial intima accumulation of 24S-HC and 27-HC is associated with advanced atherosclerotic disease and systemic inflammatory activity in individuals with severe PAD (Virginio et al. 2015).

5.2. Cancer

It is widely accepted that cells in the tumor microenvironment can be reprogrammed by tumor-derived metabolites (Villalba et al. 2013; Bovenga et al. 2015). Oxysterols have been shown to favor tumor growth either directly, by promoting cell growth, or indirectly, by dampening antitumor immune responses. It is also known that tumor-derived oxysterols recruit neutrophils endowed with pro-tumoral activities, such as neoangiogenesis (Raccosta et al. 2013).

Soncini and collegues (Soncini et al. 2016) explored the particular role played by the oxysterol 24S-HC in pancreatic tumors. They demonstrated that hypoxia inducible factor-1α (HIF-1α) controls

the abnormal expression of CYP46A1 and the generation of 24S-HC in a pancreatic neuroendocrine tumor model commonly used to study neoangiogenesis. The activation of the HIF-1α/24S-HC axis ultimately leads to the induction of the angiogenic switch through the positioning of pro-angiogenic neutrophils in proximity to CYP46A1 positive islets. Remarkably, pharmacologic blockade or genetic inactivation of oxysterols dampens the 24S-HC/neutrophils conection, controlling tumorigenesis. In samples of subjects with pancreatic neuroendocrine tumor, CYP46A1 transcripts are highly expressed, which correlates with the levels of the HIF-1α target vascular endothelial growth factor (VEGF) and tumor diameter.

A prospective clinical study in breast cancer patients receiving tamoxifen or aromatase inhibitors, in adjuvant or metastatic settings, evaluated 11 different circulating oxysterols, before and after 28 days of treatment (Dalenc et al. 2017). Tamoxifen therapy, but not the treatment with aromatase inhibitors, decreased the blood levels of 24S-HC, 7α-HC and 25-HC (a tumor promoter), but increased the levels of 4β-HC. The concentration of 27-HC increased in response to aromatase inhibitors, but not tamoxifen treatment. According to these results, the possibility of using specific oxysterols as biomarkers of tamoxifen and aromatase inhibitors' efficacy emerges as a suitable possibility.

Finally, a recent study identified a dysregulated CYP46A1/24S-HC axis in glioblastoma multiforme (GBM), the most common primary malign brain tumor in adults (Han et al. 2020). CYP46A1 expression was found to be reduced in GBM samples, compared with normal brain tissue, and the reduction in this enzyme was associated to increasing tumor grade and poor prognosis. Ectopic expression of CYP46A1 suprressed cell proliferation and tumor growth by increasing 24S-HC levels. Remarkably, treatment of GBM-derived cells with this oxysterol inhibited tumor growth, through modulation of LXR and SREBP signaling, and administration of the CYP46A1 activator efavirenz inhibited GBM growth in xenografted mice.

Conclusion

Although the consequences of variations in the the cellular cholesterol content have been extensively investigated in the context of brain physiology and pathology (Sodero et al. 2011; Ledesma et al. 2012; Martín et al. 2014), the specific cellular effects of 24S-HC represent an

emerging research field. Three important effects of 24S-HC came out from the here reviewed studies: its ability to modify cell survival, different aspects of neurotramsission, and the processing of the AD-related protein APP. Concerning the effects of 24S-HC on cell viability, middle to high concentrations of this oxysterol (above 10 μM) have been shown to promote apoptosis or necrosis, even a combined form of cell death identified as necroptosis, while low concentrations of 24S-HC (below 10 μM) induce protective effects against different types of pro-death stimuli. Because the low 24S-HC concentrations that have been tested in different in vitro assays are imilar to those found in the human brain, one may assume that the role of this oxysterol in vivo is to render protection. On the other hand, that capability of 24S-HC to modulate the activity of NMDA receptors (Paul et al. 2013) and the recycling of synaptic vesicles at excitatory synapses (Kasimov et al. 2007) stresses the possibility that certain memory deficits observed in neurological disorders may be the consequence of an abnormal cholesterol homeostasis in the brain.

Most of the studies in vitro implicate the nuclear LXR as the main receptor able to interact with 24S-HC and promote substantial transcriptional changes. Surprisingly, in mice that overexpress CYP46A1 and reach 24S-HC levels two-fold higher than the normal ones, several LXR-regulated genes normally affected in vitro, do not change their transcriptional activity (Abildayeva et al. 2006). Additional efforts are needed to identify other putative receptors for 24S-HC able to modify the performance of different brain cells. Interestingly, it has recently been demonstrated that 24S-HC, in the sub-μM range, is able to modulate the activity of adrenergic receptors in isolated atria (Odnoshivkina et al. 2019). This finding opens the possibility that the brain-derived 24S-HC that reaches the circulation may also have an impact on heart and other organs' functions.

Concerning the possibility of using 24S-HC as a biomarker in AD, the published reports only sustain the measurement of this oxysterol in CSF as a diagnosis tool, although the power of this determination is similar to, or even lower, than the power of the typical biomarkers total-tau, phospho-tau, and Aβ42. In addition, the measuremet of 24S-HC levels is not absolutely specific for AD. Variations in this metabolite have also been detected in other types of milder cognitive declines. Importantly, the fact that the concentration of 24S-HC is increased in the CSF of individuals with MCI suggests that a similar increase may also occur early in the prodromal phase of sporadic AD. Conversely, in advanced AD cases, the plasma concentration of 24S-HC descreases, most likely

reflecting the death of CYP46A1-positive neurons (see Table 1). In HD patients, a clear reduction in the 24S-HC concentration in plasma is detected at all disease stages, with subjects closer to the onset of motor signs having lower levels than those far from the onset. In congruence with the clinical studies, pre-clinical studies in mouse models of HD identified a repression of cholesterol synthesis and lower levels of striatal 24S-HC before the appearance of motor dysfunction (Leoni et al. 2008; Leoni et al. 2013; Kreilaus et al. 2015). In PD, the measurement of the 24S-HC concentration in the CSF but not plasma is a good indicator to follow the progression of the disease (Björkhem et al. 2013). In the autoimmune MS, where demyelinization is a hallmark, the determination of the 24S-HC concentration in plasma or CSF is useful to monitor the evolution of the neurodegenerative process (Leoni et al. 2002; Teunissen et al. 2003; van de Kraats et al. 2014).

The activation of 24S-HC production using low doses of the antiviral efavirenz is emerging as a promising therapeutic approach. Preclinical studies have identified improvements in mouse models of AD and GBM after efavirenz administration (Mast et al. 2017; Petrov et al. 2019; Han et al. 2020). At present, there is only one placebo controlled clinical trial recruiting subjects with MCI/early dementia due to AD to test the effects of efavirenz (https://www.clinicaltrials.gov/ct2/show/NCT03706885?term=efavirenz&cond=Alzheimer+Disease&draw=2&rank=1).

In summary, 24S-HC is not only the main by-product of brain cholesterol catabolism; instead, it is a potent bioactive molecule with therapeutic implications, and potential to be used as a biomarker in different neurological disorders.

Conflict of interest

The author declares no conflict of interests.

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--Human subjects --

Involves human subjects:

If yes: Informed consent & ethics approval achieved:

=> if yes, please ensure that the info "Informed consent was achieved for all subjects, and the experiments were approved by the local ethics committee." is included in the Methods.

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Figure legends

Figure 1. Impact of the neuron-derived 24S-HC on the neurovascular unit. The metabolite 24S-HC (orange spheres) is mostly produced in neurons by CYP46A1 activation, using cholesterol as substrate (yellow spheres). Due to the low cholesterol synthesis rate in mature neurons, the neuronal requirements of this lipid are satisfied through LRP1/LDLR-mediated import of cholesterol-containing lipoprotein particles. These particles are extracellularly asambled with cholesterol and apolipoproteins synthetized in astrocytes; astocytic cholesterol is exported to the extracellular milieu by ABC transporters. The formed 24S-HC can remain inside the neurons, diffuse into the nucleus and activate LXRs, to induce transcriptional changes. This soluble oxysterol can also cross different cell membranes to reach the nuclei of the other integrants of the neurovascular unit (astrocytes, pericytes and vascular cells). Alternatively, because the neuronal cholesterol is concentrated at the plasma membrane, the formation and entrapment of 24S-HC in the internal and external leaflets of the plasma

membrane may affect their biophysical properties, altering the processing of transmembrane substrates that are enzymatically cleaved, like the AD-related protein APP.

ABC: ATP-binding cassette transporter; LP: lipoprotein; LRP1: low density lipoprotein receptor-related protein 1; LDLR: low-density lipoprotein receptor; CYP46A1: Cytochrome P450 46A1; 24S-HC: 24S-hydroxycholesterol.

Table 1. Variations in the 24S-HC and cholesterol levels in Alzheimer disease and other forms of dementia.

AD: Alzheimer disease; VD: vascular dementia; MCI: mild cognitive impairment; LOAD: late-onset AD; SCI: subjective cognitive impairment; MCI-AD: MCI with later progression into AD; 24S-HC: 24S-hydroxycholesterol; chol.: cholesterol (total); 27-HC: 27-hydroxycholesterol; Aβ42: amyloid β peptide (of 42 aminoacids); Apo-E: apolipoprotein E; OC: occipital cortex; FC: frontal cortex; BG: basal ganglia; ↑: increased; ↓: decreased; nc: no change.

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Report	*Type of dementia*	*Plasma 24S-HC*	*CSF 24S-HC*	*Brain 24S-HC*	*Plasma chol.*	*CSF chol.*	*Other biomarkers*
Lütjohann D. et al., 2000	AD & VD	↑			nc		
Papassotiropoulos A. et al., 2000	Severe AD	↓			nc		
Bretillon L. et al., 2000	AD	↓					Plasma 27-HC: nc
Papassotiropoulos A. et al., 2002	AD, VD & MCI	nc	↑		nc	nc	
Schonknecht P. et al., 2002	AD	nc	↑		nc	nc	
Heverin M. et al., 2004	AD			↓ (OC)			Brain 27-HC: ↑ (OC, FC & BG)
Kölsch H. et al., 2004	AD, VD & MCI	↓					Plasma 27-HC: ↓
Zuliani G. et al., 2011	LOAD VD	↑ ↓			nc nc		
Popp J. et al., 2012	AD	↑	nc		nc	↓	CSF Aβ42: ↓
Leoni V. et al., 2013	SCI, MCI, MCI-AD & AD		↑			↑	CSF Apo-E: ↑ CSF Aβ42: ↓ (in MCI-AD & AD)
Li L. et al., 2018	AD	↑					Plasma Aβ42: ↑