



Cholesterol 24-Hydroxylation by CYP46A1: Benefits of Modulation for Brain Diseases

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Abstract

Cholesterol 24-hydroxylation is the major mechanism for cholesterol removal from the brain and the reaction catalyzed by cytochrome P450 46A1 (CYP46A1), a CNS-specific enzyme. This review describes CYP46A1 in the context of cholesterol homeostasis in the brain and summarizes available experimental data on CYP46A1 association with different neurologic diseases, including the mechanisms by which changes in the CYP46A1 activity in the brain could be beneficial for these diseases. The modulation of CYP46A1 activity by genetic and pharmacologic means is also presented along with a brief synopsis of the two clinical trials that evaluate CYP46A1 as a therapeutic target for Alzheimer's disease as well as Dravet and Lennox–Gastaut syndromes.

Key Words CYP46A1 · Cholesterol · 24-Hydroxycholesterol · Cholesterol turnover · Efavirenz · Neurodegeneration

Abbreviations

24HC	24-Hydroxycholesterol
ACAT1	Acyl-coenzyme A:cholesterol acyltransferase 1
AD	Alzheimer's disease
APOA1	Apolipoprotein A1
CSF	Cerebrospinal fluid
DHCR7	7-Dehydrocholesterol reductase
DHCR24	Δ 24-Dehydrocholesterol reductase
HD	Huntington's disease
LCAT	Lecithin:cholesterol acyltransferase
LXRs	Liver X receptors
MWM	Morris water maze
NMDARs	<i>N</i> -Methyl-D-aspartate receptors
NPC1	Niemann-Pick C1 protein
NPC2	Niemann-Pick C2 protein
PD	Parkinson's disease

SOAT Sterol O-acyltransferase

Introduction

Currently, there are no disease-modifying treatments for Alzheimer's disease (AD), Huntington's disease (HD), and Parkinson's disease (PD) with all of the therapies being symptomatic. Hence, different targets and pathways are now evaluated for development of disease-modifying treatments for these progressive and debilitating conditions. Among them are the studies of cytochrome P450 46A1 (CYP46A1), the cholesterol 24-hydroxylase and important brain enzyme.

Essentials of Cholesterol Homeostasis in the Brain

The brain is rich in cholesterol, a key component of eukaryotic membranes and a precursor of different neurosteroids as well as oxysterols. Cholesterol in the brain is mainly present in the two pools: in myelin sheaths of white matter, which insulate axons and enhance propagation of action potential, and in the membranes of glial cells and neurons of gray matter, which are essential for cognitive functions. In mice, these pools contain ~70% and ~30% of cholesterol, respectively, with the

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predicted cholesterol distribution in the second pool being ~20% in glial cells and ~10% in neurons (reviewed in Dietschy and Turley [1]). Cholesterol in myelin sheaths is likely metabolically stable, whereas cholesterol in cellular membranes is metabolically active with the overall rate of cholesterol turnover in the CNS being 0.03% per day (~9.1 years) in humans and 0.4% per day (~0.7 year) in mice (reviewed in Dietschy and Turley [2]). Studies in mice indicate that the rates of cholesterol turnover vary with age and cell type and in adult brain could be as high as ~30% per day in a subset of metabolically active neurons and much lower (~0.16% per day) in the remaining cells [3].

Normally, cholesterol cannot cross the blood–brain barrier; therefore, cholesterol maintenance in the brain includes *in situ* biosynthesis (almost the only source of cholesterol), cholesterol transport between different cells, and elimination (Fig. 1). Cholesterol biosynthesis, a complex process involving multiple steps, proceeds in the brain via the two major pathways: the Bloch pathway, which is suggested to take place in astrocytes, and the Kandutsch–Russell pathway, which seems to be operative in neurons (reviewed in Pfrieger and Ungerer [4]). Both pathways are important for brain physiology because genetic deficiencies of $\Delta 24$ -dehydrocholesterol reductase (DHCR24) and 7-dehydrocholesterol reductase (DHCR7) catalyzing the last steps in the Bloch and

Kandutsch–Russell pathways, respectively, lead to desmosterolosis and Smith–Lemli–Opitz syndrome, respectively, the rare hereditary diseases characterized by neurological problems [5, 6]. In the brain, the capacity of different cells to synthesize cholesterol varies and depends on age; the glial cells (especially astrocytes) are believed to synthesize most of the brain cholesterol in adults. This cholesterol is then delivered to neurons to satisfy their high demand for cholesterol, probably for building the extensive membrane surface of axons, dendrites, and synapses as well as formation of synaptic vesicles (reviewed in Pfrieger and Ungerer [4] and [7]).

To deliver cholesterol to neurons, astrocytes synthesize apolipoproteins, mainly E (APOE), J (APOJ or clusterin), and D (APOD), which after secretion serve as acceptors for astrocytic cholesterol effluxed by the ABC transporters (mainly by ABCA1). The secreted APOE and APOJ could bind cholesterol and other lipids to form the HDL-like lipoprotein particles, whereas APOD binds lipids and associates with other lipoproteins (reviewed in Pfrieger and Ungerer [4], [8], [9], and Elliott et al. [10]). The astrocyte-produced apolipoprotein particles reach neurons and other cells and bind to the cell surface receptors. The prototypic low-density lipoprotein receptor (LDLR, expressed on both glial and neuronal cells) and the LDLR-related

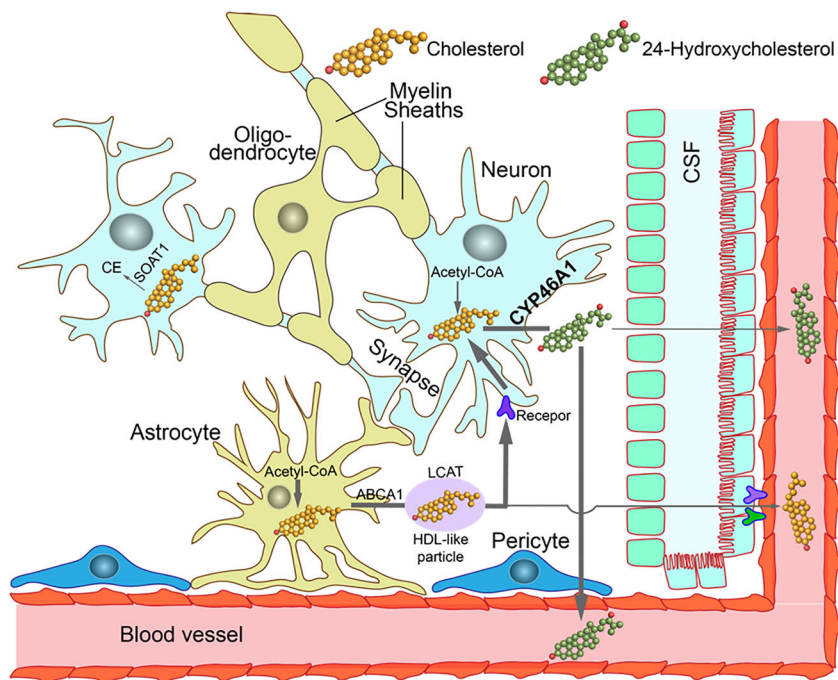


Fig. 1 Major pathways of cholesterol homeostasis in adult brain. Arrow thickness indicates the relative quantitative significance of the pathway in humans. Cholesterol (shown as a ball model in orange, the hydroxyl group is in red) is synthesized from acetyl-CoA in both neurons and astrocytes, and then could be effluxed from astrocytes by ABCA1 and transported on high-density lipoprotein (HDL)-like particles to neurons where it is taken up by a receptor-dependent mechanism. Once in neurons, CYP46A1 can convert cholesterol excess to 24-hydroxycholesterol

(24HC, shown as a ball model in green, the hydroxyl groups are in red), which could rapidly diffuse to the CSF and then through the blood–brain barrier to the systemic circulation for delivery to the liver. Some of the HDL-like particles could travel to the cerebrospinal fluid (CSF) and then gain access to the systemic circulation via the receptor-mediated endocytosis. A small portion of cholesterol could be esterified by sterol-O-acetyltransferase 1 (SOAT1) to form cholesterol esters (CE)

protein 1 (LRP1, predominantly expressed in neurons) are the two major receptors that are probably involved in cholesterol uptake by brain cells. The brain also expresses other receptors, including the very-low-density lipoprotein receptor (VLDLR), the APOE receptor 2 (APOER2 or LRP8), and megalin (LRP2), that can bind lipoprotein particles (reviewed in Pfrieger and Ungerer [4], Arenas et al. [11], and Bjorkhem and Meaney [12]). Remarkably, recently, an individual with a total lack of APOE was identified and found to have normal cognitive and neurological functions as well as normal brain structure on magnetic resonance imaging [13]. This clinical finding suggested that the APOE functions are redundant and other apolipoproteins in the brain can compensate for APOE absence.

Once taken up by a cell, cholesterol is delivered to late endosomes/lysosomes, in which the Niemann-Pick C1 (NPC1) and C2 (NPC2) proteins bind cholesterol and mediate its export to endoplasmic reticulum and plasma membranes. The importance of the endolysosomal cholesterol traffic in the brain is highlighted by the mutations in NPC1 and NPC2, which lead to Niemann-Pick type C disease. This is a lysosomal storage disorder characterized by cholesterol accumulation in the endolysosomes of all the tissues with, however, the most devastating consequence of this accumulation being in the brain, which develops neurodegeneration and neuronal abnormalities (reviewed in Vance and Karten [14]).

To maintain the steady-state levels, cholesterol biosynthesis in the brain is balanced by cholesterol elimination. One mechanism is cholesterol hydroxylation by the cytochrome P450 enzymes: ubiquitous CYP27A1 [15], steroidogenic CYP11A1 [16], and CNS-specific CYP46A1 [17], which convert cholesterol to 27-hydroxycholesterol, pregnenolone, and 24S-hydroxycholesterol (24HC), respectively. Of these enzymatic reactions, cholesterol 24-hydroxylation by CYP46A1 is quantitatively significant and was found to account for elimination of 75–85% and 40–50% of cholesterol excess from human and mouse brains, respectively [18, 19]. Unlike membrane-impermeable cholesterol, 24HC could rapidly diffuse out of cells and gain access to the systemic circulation (~98%) or cerebrospinal fluid (CSF, ~2%) for delivery to the liver for further degradation to bile acids [20, 21]. CYP46A1 is a microsomal enzyme, expressed preferentially in the brain and to a much lower extent in the retina [17, 22, 23]. In the brain, CYP46A1 is found in pyramidal neurons of the hippocampus and cortex, and Purkinje cells of the cerebellum as well as hippocampal and cerebellar interneurons [24–26]. In both humans and mice, the brain CYP46A1 levels rapidly increase after birth and reach a steady state by 1 year in humans (and 2–4 weeks in mice) [17], suggesting CYP46A1 importance for brain functions in adults.

Studies in mice indicate that changes in the rate of cholesterol 24-hydroxylation lead to compensatory decreases or increases in the rate of cholesterol biosynthesis by yet an unknown mechanism, and thereby suppress or stimulate brain cholesterol turnover [18, 27–29]. Because of the high CYP46A1 expression in the brain, serum 24HC levels were found to be a biomarker of the enzyme activity in the brain and reflect the ratio of the brain weight to liver volume [17, 30]. Apolipoprotein-mediated transport through the CSF is the other mechanism by which the brain clears cholesterol excess. If cholesterol 24-hydroxylation removes most and approximately a half of cholesterol excess from human and mouse brains, respectively [18, 19], then apolipoprotein-mediated transport through the CSF could lead to up to 15–25% and 50–60% of the brain cholesterol elimination in humans and mice, respectively. The details of this mechanism are still under investigation but likely involve apolipoprotein A1 (APOA1) as APOA1-containing particles could diffuse to the CSF and then be taken up via endocytosis by the receptors (LRP1 and scavenger receptor class B type 1) that are expressed on the brain capillary endothelial cells (reviewed in Pfrieger and Ungerer [4] and Zhang and Liu [31]). In addition to cholesterol elimination, a small excess of brain cholesterol is esterified by ACAT1 (acyl-coenzyme A:cholesterol acyltransferase 1) or SOAT1 (sterol O-acyltransferase 1), which resides in the endoplasmic reticulum and acts on intracellular cholesterol ([32, and reviewed in Chang et al. [33]). LCAT (lecithin:cholesterol acyltransferase) is another cholesterol-esterifying enzyme in the brain, which is secreted by astrocytes and esterifies cholesterol on nascent APOE-containing particles [34]. In healthy adult humans and mice, cholesterol esterification is of very minor significance because cholesterol esters constitute only about 1% of total brain cholesterol (reviewed in Bjorkhem and Meaney [12]). Thus, of the mechanisms which target cholesterol excess in the brain, cholesterol removal by CYP46A1 is of key importance; this mechanism represents not only the major pathway of cholesterol elimination but also the means to control brain cholesterol biosynthesis and turnover.

CYP46A1 Activity Modulation for Major Diseases of the Brain

This section will describe the three major mechanisms by which modulation of CYP46A1 activity in the brain could affect brain processes and function, namely by changing: 1) the steady-state levels of cholesterol; 2) the levels of 24HC, a biologically active molecule; and 3) the rate of brain cholesterol turnover (Fig. 2).

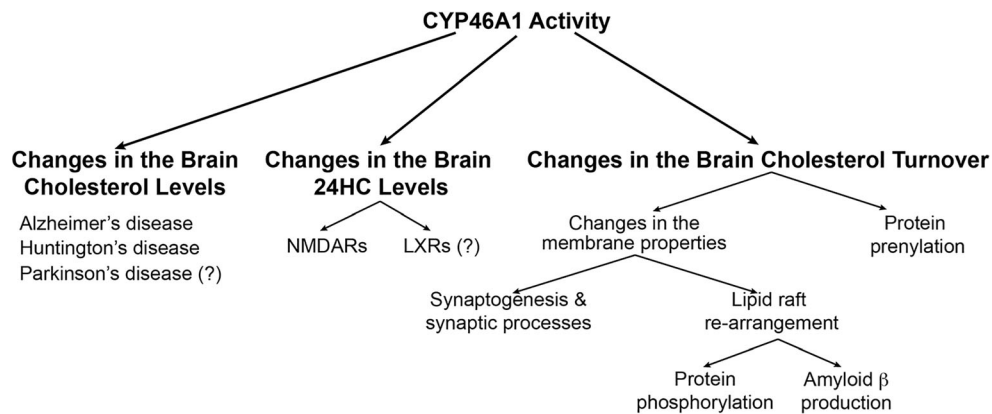


Fig. 2 Major mechanisms whereby modulation of CYP46A1 activity can affect brain processes. The three major mechanisms are changes in the brain cholesterol levels, 24-hydroxycholesterol (24HC) levels, and cholesterol turnover. The brain cholesterol levels could modulate the progression of several neurodegenerative disorders. The 24HC cholesterol levels can affect the activation of *N*-methyl-D-aspartate receptors (NMDARs) involved in memory and cognition and the activation of liver X receptors (LXRs), important transcription factors controlling gene expression in multiple pathways. The brain cholesterol

turnover can alter physicochemical properties of plasma and subcellular membranes and thereby synaptogenesis and synaptic processes as well as lead to lipid raft rearrangement. The latter can in turn alter phosphorylation and amyloid β production. In addition, the brain cholesterol turnover can determine the rate of cholesterol biosynthesis and hence the production of non-sterol isoprenoids required for protein prenylation. The question marks indicate the lack of sufficient experimental evidence

CYP46A1 and Brain Cholesterol Levels in AD, HD, and PD

CYP46A1 was first linked to the diseases of the brain by the measurements of plasma 24HC levels. The levels of this sterol were slightly increased in the plasma of patients with early AD and non-AD demented patients but were unchanged in patients with depression [35]. Conversely, patients with advanced AD had decreased 24HC levels in their plasma, likely reflecting the marked destruction of the CNS and hence a reduction in the CYP46A1 levels and the flux of 24HC from the brain [36, 37]. In agreement with this explanation, the measurements of 24HC in the four brain regions (the frontal and occipital cortexes, basal ganglia, and pons, Fig. 3) of patients with AD revealed decreased sterol levels in all four regions and a close coupling between cholesterol biosynthesis and elimination [38]. Simultaneously, the brain of patients with AD was discovered to have a different CYP46A1 distribution as compared to that in control subjects; in addition to neurons, some of the astrocytes in the AD-affected brain were found to express CYP46A1 [24, 39]. Because AD leads to synaptic and then neuronal loss and thereby a decrease in the CYP46A1 content, CYP46A1 expression in astrocytes was suggested to compensate for the CYP46A1 decrease due to neurodegeneration [39]. Notably, *CYP46A1* was revealed to be a highly polymorphic gene with the frequency of some of its intronic polymorphism being as high as 40% (<https://www.ncbi.nlm.nih.gov/snp/>). Some but not all genetic studies linked the CYP46A1 intronic polymorphisms to AD (reviewed in Russell et al. [26]), yet the current meta-analyses suggest that only some of

the CYP46A1 polymorphisms could be associated with AD albeit being minor risk factors and ethnicity specific [40–42].

Unfortunately, there have been no systematic investigations of the absolute cholesterol levels in different brain regions of patients with AD. Available data are scarce and equivocal [43, 44]. Perhaps the most comprehensive was the study on AD samples that evaluated the frontal and occipital cortexes, basal ganglia, and pons not only for the levels of different sterols but also for cholesterol content, which was increased only in the basal ganglia [38]. In a different study, cholesterol was found to be increased in the gray matter frontal cortex of the AD carriers of the APOE4 genotype as compared to the APOE4 control subjects [45]. Conversely, the cholesterol levels in crude hippocampal membranes from the nongenotyped AD patients were reported to have only minor changes [46]. Consistent with this report are the data on unchanged cholesterol levels in the temporal and frontal cortexes affected by AD, and decreased cholesterol levels in the lipid raft fraction from the temporal but not frontal cortex [47]. Furthermore, local cholesterol accumulation was detected in the amyloid β -positive presynaptic nerve terminals from the AD cortex [48] as well as in the core of mature, but not diffuse, amyloid plaques [49]. In contrast, cholesterol levels in white matter were found to be reduced in AD patients, likely as a result of extensive axonal demyelination [50]. Thus, available literature data suggest that there are probably region- and membrane-specific changes in the brain cholesterol levels in AD. Also, epidemiologic studies established that the $\epsilon 4$ allele of APOE and variants of APOJ are risk factors for late-onset AD [51–54], which in part could be due to the altered capacity of the brain apolipoprotein particles to transport and deliver

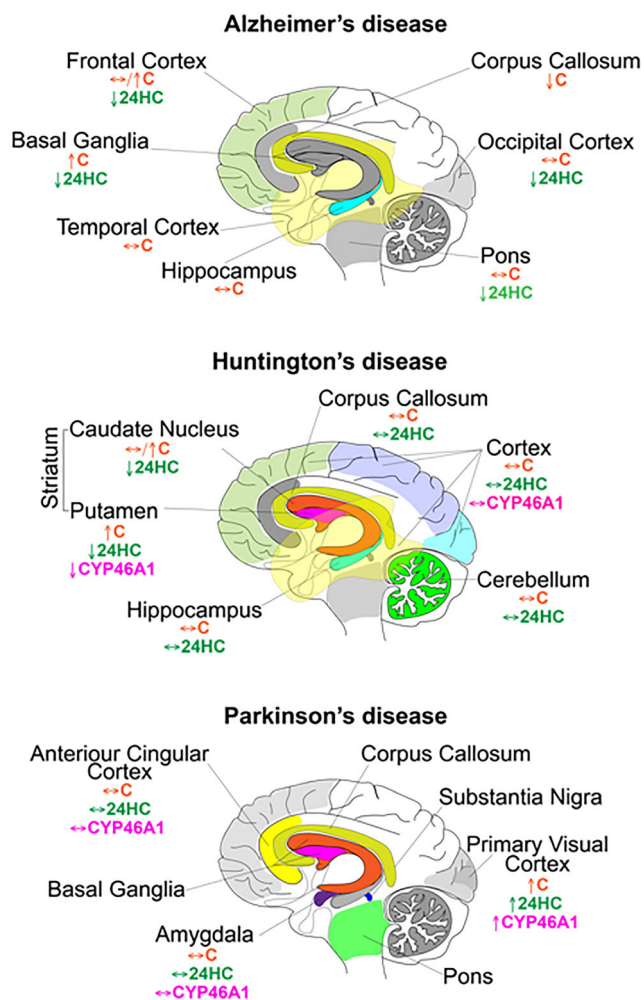


Fig. 3 Brain regions affected by Alzheimer's, Huntington's, and Parkinson's diseases. The regions in color on sagittal sections are those that are mostly affected by the disease; the regions in gray are those that are affected to a lesser extent or at a later disease stage. Upward arrows, downward arrows, and left-right arrows indicate increase, decrease, and no change, respectively, in the levels of cholesterol (C, in orange) and 24-hydroxycholesterol (24HC, in green), and CYP46A1 (in magenta) in different regions of the disease-affected brain as discussed in the main text

cholesterol (reviewed in Mahley and Rall [55], Mahley [56], and Bu [57]). Accordingly, increase in the CYP46A1 expression or activity in the brain may be beneficial for AD to compensate for changes in the brain apolipoprotein-mediated transport.

HD is an autosomal dominant neurodegenerative disorder due to the mutation in the huntingtin gene [58]. This disease is characterized by cell loss and astrogliosis in several brain regions with the most prominent alterations occurring in the striatum (comprised of caudate nucleus and putamen, Fig. 3), ultimately leading to striatal atrophy [59]. Plasma 24HC levels were shown to be reduced in HD patients and correlate with disease severity and markers of disease progression [60–62]. This reduction is consistent with the reported decreases in the 24HC levels in the caudate and putamen as well

as the reduction of the CYP46A1 levels in the putamen of the HD patients [63, 64]. Also, of the four studied regions in HD samples (putamen, caudate, gray cortex, white cortex, and cerebellum), only the putamen was found to have increased cholesterol levels as well as the levels of the major cholesterol precursors [64]. This is in contrast to cholesterol increase in the caudate of HD patients in a different study [65], and the suggested impairment in cholesterol biosynthesis in the cortex and striatum of HD patients in a study that quantified the levels of HMGCR, CYP51, and DHCR7 involved in cholesterol biosynthesis [66]. Thus, available data are not consistent; nevertheless, they all suggest that cholesterol homeostasis is altered in the HD-affected brain and that CYP46A1 could be a reasonable therapeutic target for this disease.

Finally, PD, a common neurodegenerative disorder, is characterized by a marked loss of dopaminergic neurons in the pars compacta of the substantia nigra (a region of the midbrain, Fig. 3) as well as the formation of Lewy bodies, which are composed of protein aggregates, mainly those of α -synuclein (reviewed in Lee and Trojanowski [67]). So far, three studies measured the 24HC levels in PD patients. In one study, no changes in plasma levels of total 24HC were found in patients with PD, thus suggesting that CYP46A1 is not affected in this disorder. Interestingly, there was a significant correlation between the levels of 24HC in the patient CSF and disease duration, raising a possibility that this CSF oxysterol may be of value to follow the disease progression [68]. In a different study, which only measured the plasma levels of unesterified 24HC, the PD group had a decrease in the plasma content of this oxysterol [69]. The third study showed that plasma levels of esterified 24HC are significantly reduced in patients with PD and could reflect an impaired catalytic activity of LCAT as result of a higher level of oxidative stress in the CNS. Esterified 24HC in plasma was suggested to be a novel biomarker of neurodegeneration in PD as well as a measure of disease severity and progression [70]. In addition, several brain regions in patients with PD were characterized by a comprehensive lipidomics analysis: these were the primary visual cortex (a region that is mainly devoid of structural but not functional changes) and the amygdala and anterior cingulate cortex (two regions which contain Lewy bodies at the different disease stages but do not have such severe degeneration as the substantia nigra) [71]. In the PD samples, cholesterol levels were only increased in the visual cortex but not amygdala and anterior cingulate cortex. Similarly, the visual cortex had an increase in the 24HC levels and an increase in the levels of CYP46A1 [71]. Lastly, both statins (inhibitors of cholesterol biosynthesis) and higher serum cholesterol were suggested to reduce PD risk, although for both conflicting findings were reported [72–78]. A recent meta-analysis showed that a protective effect of statins was observed in studies that did not adjust for serum cholesterol and was not observed in studies that performed this adjustment [73].

Additional investigations are required to make definite conclusions about the benefit of CYP46A1 activity modulation in PD.

Brain Effects of 24HC

Modulation of CYP46A1 activity changes the brain levels of 24HC (Fig. 2), a known activator/modulator of at least two receptors—liver X receptors (LXRs) and *N*-methyl-D-aspartate receptors (NMDARs). LXRs are the transcription factors and master regulators of cholesterol elimination; they are also involved in the regulation of fatty acid and triglyceride biosynthesis and glucose metabolism as well as immune-inflammatory responses (reviewed in Kalaany and Mangelsdorf [79], Glass and Ogawa [80], and Calkin and Tontonoz [81]). LXR activation by synthetic LXR agonists was shown to be neuroprotective and anti-inflammatory in mouse models of AD, PD, traumatic brain injury, stroke, and amyotrophic lateral sclerosis (reviewed in Courtney and Landreth [82]). However, there is no direct evidence that LXRs are activated by 24HC in the brain as the brain expression of LXR target genes was not increased in heterozygous transgenic mice, which overexpressed *CYP46A1* and had about a 2-fold increase in their brain 24HC levels [83]. Possibly, a higher than a 2-fold increase in the 24HC levels is necessary to activate LXRs in the brain. Alternatively, rapid diffusion of the 24HC surplus out of cells [21] precludes the oxysterol binding to LXRs, which mainly reside in the nucleus but in some cell types can also be found in the cytoplasm or plasma membranes [84, 85].

24HC is a positive allosteric modulator of NMDARs, which mediate excitatory neurotransmission throughout the CNS and are crucial for synaptic plasticity and learning [86–88]. Specifically, 24HC was found to act mainly on NMDARs which contain the GluN2B subunit [89], an important target for NMDAR modulation by ketamine and memantine, the latter being a medication approved by the FDA to ameliorate the symptoms of AD [90, 91]. An increase in 24HC as a result of CYP46A1 activation could enhance the Ca^{2+} current through NMDARs and improve memory and learning. Also, CYP46A1 activation could be useful when there is a decrease in glutamatergic synaptic transmission, for example in schizophrenia or in overdose with ketamine and phencyclidine, NMDAR antagonists and recreational drugs (reviewed in Xia et al. [92] and Coyle [93]). However, overstimulation of NMDARs leads to excitotoxicity and neuronal cell death and was found in such pathologic conditions as epilepsy and hyperalgesia (reviewed in Collingridge et al. [86]). Accordingly, CYP46A1 should be inhibited in the conditions of NMDAR overstimulation. Of importance is that excessive stimulation of glutamate receptors in mice was shown to induce a loss of membrane cholesterol due to

metabolism by CYP46A1 [94], thus creating a vicious cycle of NMDAR overstimulation. Remarkably, *in vitro*, CYP46A1 was also shown to be activated by glutamate, raising a possibility of a reciprocal relationship between CYP46A1 activity and neurotransmission: increased CYP46A1 activity enhances neurotransmission, and excitatory neurotransmission in turn increases CYP46A1 activity [95]. *In vivo* studies are required to test this possibility and clearly establish the role of 24HC. Unfortunately, there have yet been no *in vivo* investigations unambiguously showing uncoupling between changes in the brain 24HC levels and cholesterol turnover, i.e., when the 24HC levels were changed but the rate of cholesterol turnover remained unaffected as indicated by unchanged levels of lathosterol and desmosterol (as markers of the neuronal and astrocytic cholesterol biosynthesis, respectively, reviewed in Pfrieger and Ungerer [4]) and either unchanged or reduced levels of the brain total cholesterol.

Brain Effects of the Altered Cholesterol Turnover

Similar to studies of the individual 24HC effects, the investigation of the individual effects of brain cholesterol turnover is difficult because of the tight link to the 24HC levels. Hence, this section will only consider how cholesterol turnover could affect the membranes and membrane-associated processes in the brain (Fig. 2) by changing the flux of cholesterol and 24HC through the membranes and lipid rafts in the regions where CYP46A1 is expressed. Cholesterol is abundant in plasma membranes, where it regulates the physicochemical properties of the membranes (e.g., membrane fluidity, thicknesses, and permeability). Also, cholesterol serves as a glue for the organization of the membrane microdomains called lipid rafts, which are important for cellular signaling and function of a number of membrane proteins (reviewed in Sezgin et al. [96] and Grouleff et al. [97]).

Perhaps the most prominent effect of the cholesterol turnover could be on synaptic processes and higher-order brain functions; cholesterol was shown to be important for synaptogenesis, virtually all aspects of synaptic transmission (neurotransmitter release, synaptic vesicle traffic, and postsynaptic receptor clusterization) as well as synaptic plasticity [98–102]. This effect will be discussed in the next sections, which will summarize the key findings from the characterizations of mice with altered cholesterol turnover. In addition, changes in the brain cholesterol turnover could lead to lipid raft rearrangement and subsequent alteration of protein phosphorylation and generation of amyloid β . The former point is indirectly supported by a study of the brain phosphoproteome of *Cyp46a1*^{-/-} mice, which showed a general decrease in protein phosphorylation [103], possibly as a result of altered targeting of protein kinases to their protein substrates and vice versa protein substrates to their kinases, which reside in lipid rafts.

The latter point is supported by enrichment of lipid rafts with amyloid precursor protein and the secretases that cleave this protein and generate amyloid β peptides. There are numerous cell culture studies, which link membrane cholesterol and the amyloid β generation, yet *in vivo* effects of the brain cholesterol turnover have not yet been demonstrated, except only in one study, which convincingly related cholesterol turnover to protein prenylation [104] as described below.

Modulation of CYP46A1 Activity by Genetic Means

CYP46A1 transcription was found to be insensitive to major regulatory axes, except oxidative stress [105]. Studies show that *CYP46A1* may be controlled epigenetically [106–108] and by the specificity (Sp) transcription factors as the levels of basal expression [109, 110]. So far, the modulation of CYP46A1 activity at the gene level was by *Cyp46a1* ablation or *CYP46A1*-containing adenovirus injections (Table 1).

The generation of *Cyp46a1*^{-/-} mice was the first genetic manipulation of CYP46A1 activity in mammals [18]. These mice provided seminal knowledge of a role of CYP46A1 in cholesterol homeostasis in the brain and linked cholesterol 24-hydroxylation and cholesterol turnover in the brain to the higher-order brain functions [18, 26, 104, 118, 119]. A lack of

CYP46A1 was discovered to lead to severe deficiencies in spatial, associative, and motor learning as well as in hippocampal long-term potentiation [104]. The underlying reason for cognitive deficits was established as well, namely a reduced production of geranylgeraniol, a nonsterol isoprenoid required for hippocampal long-term potentiation [104, 118], synaptic basis for learning and memory. The steady-state cholesterol levels were unchanged in the brain of *Cyp46a1*^{-/-} mice because the rate of cholesterol biosynthesis was reduced almost 2-fold to compensate for *Cyp46a1* ablation [18]; hence, the production of nonsterol isoprenoids was also reduced because these compounds are generated during cholesterol biosynthesis.

The cortical and hippocampal injections of the *CYP46A1*-containing adenovirus to APP23 or APP/PS mice, the models of AD, were the first genetic manipulation which enhanced CYP46A1 activity in mammals [112]. These injections, before and after the onset of amyloid plaques, increased CYP46A1 expression and the levels of 24HC in the brain, while reducing the amyloid β pathology and rescuing some cognitive deficits [112]. This work provided the first experimental evidence that CYP46A1 could be a therapeutic target for AD. Simultaneously, genetic ablation of *Acat1* (or *Soat1*) in triple transgenic mice, which contained mutant human amyloid precursor protein, presenilin 1, and Tau protein, was found to also increase the 24HC levels, while reducing the cholesterol biosynthesis rate and amyloid pathology [32].

Table 1 A summary of mouse models used for the CYP46A1 activity modulation

Mouse genotype and/or strain	Experimental approach	References
Increased production of 24HC		
C57Bl/6NCtrl C57Bl6	<i>CYP46A1</i> transgenic mice	[27, 83]
3XTg-AD mice on the 129/C57BL/6 background	<i>Acat1</i> ablation	[32]
THY-Tau22 mice	The <i>CYP46A1</i> -containing adenovirus injection to the hippocampus	[111]
APP23 (Thy1-hAPP ^{swc}) and APP/PS mice	The <i>CYP46A1</i> -containing adenovirus injection to the cerebral cortex and hippocampus	[112]
R6/2 [B6CBA-Tg (HDexon1)62Gpb/1J]	The <i>CYP46A1</i> -containing adenovirus injection to the striatum	[63]
C57BL/6J mice	Efavirenz treatment with both CYP46A1-activating and inhibiting doses	[28]
5XFAD mice on the B6SJL background	Efavirenz treatment with the CYP46A1-activating dose	[29]
Swiss albino mice	Efavirenz treatment with the CYP46A1-activating dose	[113]
Decreased production of 24HC		
129S6/SvEv	<i>Cyp46a1</i> ablation	[18]
C57BL/6 and APP23 transgenic mice	The <i>shCyp46a1</i> -containing adenovirus injection to the hippocampus	[114, 115]
C57BL/6	The <i>shCyp46a1</i> -containing adenovirus injection to the striatum	[63]
C57/B6J	Treatment with voriconazole (a CYP46A1 inhibitor)	[116]
Swiss albino mice	Treatment with voriconazole (a CYP46A1 inhibitor)	[113]
Tg2576 mice	Efavirenz treatment with the CYP46A1-inhibiting dose	[117]

Then, hetero- and homozygous transgenic mice, which overexpressed *CYP46A1*, were generated [27, 83]. The 24HC levels and the levels of lanosterol, a cholesterol precursor, were increased in these animals indicating enhancement of cholesterol turnover. Notably, homozygous *CYP46A1* transgenic mice (15-month-old females) showed an increased expression of the studied pre- and postsynaptic proteins and improvement in spatial memory as assessed by the Morris water maze (MWM) test. This study demonstrated that increased *CYP46A1* activity and brain cholesterol turnover are of benefit for cognitive function under normal conditions [27].

In the more recent investigations, additional insights into the role of *CYP46A1* activity were obtained. When THY-Tau22 mice, an AD model of tau but not amyloid pathology, received bilateral hippocampal injections of the *CYP46A1*-containing adenovirus, the *CYP46A1* expression was selectively enhanced in the hippocampal neurons. These injections rescued mouse cognitive deficits as well as impaired long-term depression and spine defects but did not affect Tau phosphorylation and the associated gliosis. The authors suggested that *CYP46A1* may be a relevant therapeutic target for tauopathies, especially in AD [111]. Similarly, there was a beneficial effect of the adenoviral injections of *CYP46A1* in the striatum of the R6/2 mice, a HD model. These injections decreased neuronal atrophy; the number, intensity level, and size of huntingtin aggregates; and ameliorated motor deficits. Conversely, when wild-type mice received striatal injections of the virus encoding the short hairpin (sh) *Cyp46a1* RNA to reduce the gene expression, they developed the HD phenotype with spontaneous striatal neuron degeneration and motor deficits. Collectively, these and other data provided experimental evidence that *CYP46A1* could be a therapeutic target for HD [63]. Yet, when the sh*Cyp46a1* RNA was delivered to the hippocampus of wild-type mice, the cholesterol content was increased in neurons followed by cognitive deficits, enhanced production of the amyloid β peptides, abnormal tau phosphorylation, and hippocampal atrophy due to apoptotic neuronal death [114]. In a different study, which also utilized hippocampal injections of the sh*Cyp46a1* RNA to wild-type mice, similar results were obtained, and, in addition, it was shown that silencing *Cyp46a1* expression induces neuronal sclerosis and epileptic activity in mouse hippocampus [115]. Thus, modulation of *CYP46A1* activity by genetic means demonstrated that region-specific decreases of *CYP46A1* activity may lead to different pathologic consequences (AD- and HD-like pathology and epilepsy) and pointed to the diseases, which may utilize *CYP46A1* as a therapeutic target.

Pharmacologic Modulation of CYP46A1 Activity

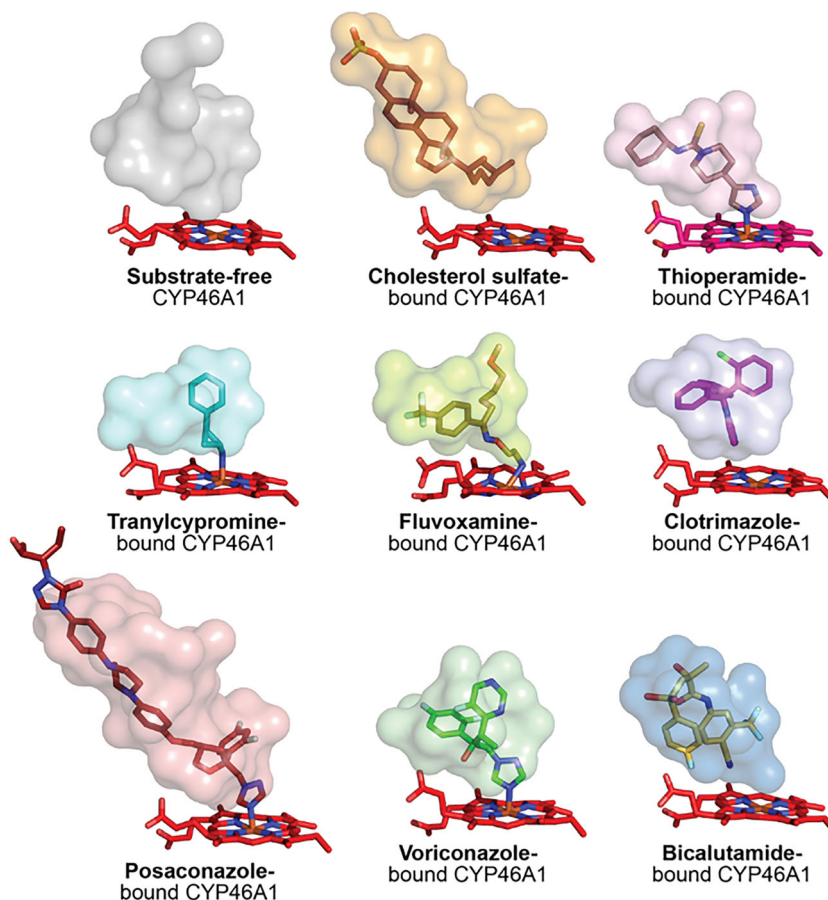
The *CYP46A1* potential as a pharmacologic target was first implicated by the biochemical characterizations of the purified

recombinant enzyme [120]. In addition to hydroxylating cholesterol and 24HC, *CYP46A1* was found to metabolize structurally diverse C21- and C27-steroids as well as some marketed drugs [120]. These data suggested that *CYP46A1* has a flexible active site and prompted structural studies of the enzyme along with the screening of the marketed drugs for the effect on *CYP46A1* activity *in vitro*. Initially, substrate-free and cholesterol substrate-bound crystal structures of *CYP46A1* were determined, which confirmed conformational flexibility of the enzyme active site (Fig. 4) [121]. Then, based on the results of drug screening, *CYP46A1* was additionally cocrystallized in complex with seven other compounds (Fig. 4), mainly *CYP46A1* inhibitors and the FDA-approved pharmaceuticals: the anticonvulsant thioperamide; the antidepressants tranylcypromine and fluvoxamine; the antifungals clotrimazole, posaconazole, and voriconazole; as well as the antineoplastic agent bicalutamide [122–125]. These structures revealed that the *CYP46A1* active site can accommodate compounds of different shapes, sizes, and polarity because of the conformational fit induced by these compounds.

A remarkable finding from the screening of > 300 FDA-approved drugs was that *in vitro* *CYP46A1* activity may not only be inhibited by different medications but also activated by agomelatine and mirtazapine (antidepressants), huperzine and galantamine (anti-AD medications), and acetaminophen and phenacetin (analgesics), as well as efavirenz (an anti-HIV drug) [121, 123]. This discovery gave impetus to animal experiments, which showed that voriconazole, the *in vitro* *CYP46A1* inhibitor, also inhibits the P450 in mouse brain when given intraperitoneally and at a clinically relevant dose [116] (Table 1). Efavirenz was chosen as a *CYP46A1* activator and was found to activate the enzyme in mouse brains at low concentrations but inhibit the P450 at higher concentrations [28]. Thus, it was proved that pharmacologic modulation of *CYP46A1* activity is possible *in vivo*. To understand the mechanism of a dual efavirenz effect on *CYP46A1* activity, studies with the purified P450 were carried out. These studies established that *CYP46A1* has the allosteric site on the protein surface, which is away from the active site located inside the protein molecule [126]. Accordingly, at a low concentration, efavirenz was suggested to bind to the allosteric site and activate *CYP46A1*, whereas at high concentrations the drug likely binds to both allosteric and active sites and inhibits *CYP46A1* because of the competition with cholesterol for the active site [28].

Efavirenz is usually given to HIV patients at the high dose of 600 mg/day, which is associated with the CNS toxicity and psychiatric events in 40–60% and 25–40% of patients, respectively (reviewed in Apostolova et al. [127]). Yet, the activating *CYP46A1* dose in mice (0.1 mg/day/kg of body weight) equaled to about 7 mg/day if given to humans, which was a much lower dose than that given to HIV patients. Hence,

Fig. 4 Plasticity of the CYP46A1 active site. The enclosed volumes of the CYP46A1 active site, substrate-free and in complex with different compounds, are shown as semitransparent surfaces. Compounds inside the active site are shown as stick models. The heme group, which is the site of catalysis in the P450 enzymes, is in red



efavirenz was tested for the effects on 5XFAD mice, an AD model, in which amyloid deposition begins at 2 months of age and behavioral deficits start to develop at 4 months of age [128]. Two efavirenz treatments were evaluated so far: the so-called 1st and 2nd treatment paradigms (Fig. 5, the results of the 2nd treatment paradigm will be published elsewhere).

In both, efavirenz was given to mice in drinking water at the 0.1 mg/day/kg of body weight dose. In the 1st treatment paradigm, efavirenz was administered from 1 month of age (before amyloid plaque appearance) to 9 months of age [29]; in the 2nd treatment paradigm, efavirenz dosing began at 3 months of age (after amyloid β deposition) and continued

Fig. 5 A comparative summary of the long-term efavirenz treatments of 5XFAD mice. Upward arrows, downward arrows, and left-right arrows indicate increase, decrease, and no change, respectively. The data are taken from Mast et al. 29 (the 1st treatment paradigm) and will be published elsewhere (the 2nd treatment paradigm)

Efavirenz Treatment (0.1 mg/kg/day)			
	1 st Treatment Paradigm		2 nd Treatment Paradigm
	1 to 5 months of age	1 to 9 months of age	3 to 9 months of age
Amyloid β measurements:	↓, ELISA ↓, 6E10 ↓, Thioflavin S	↓, ELISA	↔, ELISA ↔, 6E10 ↓, Thioflavin S
Behavioral performance:	↓, Y-maze test ↓, MWM test ↔, Learning fear test ↑, Contextual fear test ↔, Cued fear test	↔, Y-maze test ↑, MWM test ↔, Learning fear test ↔, Contextual fear test ↔, Cued fear test	↔, Y-maze test ↑, MWM test ↑, Learning fear test ↑, Contextual fear test ↔, Cued fear test
Sterols measurements:	↑, 24HC ↑, Lathosterol ↔, Desmosterol ↑, Cholesterol turnover	↑, 24HC ↑, Lathosterol ↔, Desmosterol ↑, Cholesterol turnover	↑, 24HC ↑, Lathosterol ↔, Desmosterol ↑, Cholesterol turnover

for 6 months. In both treatments, efavirenz activated CYP46A1, brain cholesterol turnover, and improved animal performance in the MWM test at 9 months of age. In addition, in the 2nd treatment paradigm, behavioral improvements were observed in the contextual fear conditioning tests. The principal difference between the two treatment paradigms was in efavirenz effects on the amyloid β load. In the 1st treatment paradigm, there were decreases in the amyloid β pathology as assessed by all the quantification methods: ELISA and stains of mouse cortex and hippocampus with Thioflavin S and 6E10, which mainly bind to the dense- and diffuse-core plaques, respectively. In contrast, in the 2nd treatment paradigm, there were no decreases in the amyloid β burden as assessed by ELISA and 6E10 stain but there were small reductions by 17–20% in the number and area of Thioflavin S-positive plaques in the cortex and hippocampus. Thus, in contrast to the 1st paradigm, which effectively decreased amyloid β load in the brain [29], the 2nd paradigm did not have such a pronounced effect on the amyloid β pathology. This difference is likely due to the lack or presence of amyloid plaques at the initiation of treatment and different treatment durations (6 or 8 months).

An interesting observation provided by the 1st treatment paradigm was that cholesterol was depleted from mouse brains during the first 3 months of efavirenz treatment. This effect was linked to the young age of mice, whose brain and pool of brain cholesterol expand until the age of 3 months (reviewed in Dietschy and Turley [1]). Accordingly, when 1-month-old mice were put on efavirenz treatment in the 1st treatment paradigm, CYP46A1 activation and enhancement of the brain cholesterol turnover slowed down their cholesterol accumulation in the brain, and this effect was observed until the age of 3 months when animals became mature. Simultaneously with cholesterol depletion, young efavirenz-treated mice had impaired performance in the Y- and Morris water maze tests, yet improved performance in the contextual fear conditioning test [29]. Therefore, changes in behavioral performance of young efavirenz-treated mice were attributed to cholesterol depletion from the brain during the first 3 months of efavirenz dosing. Nevertheless, despite transient behavioral impairments, the 1st and 2nd treatment paradigms clearly demonstrated that a long-term pharmacologic CYP46A1 activation could be beneficial for AD. These studies also showed that efavirenz effects are context-specific and served as a basis for the proof-of-concept clinical research study of efavirenz in patients with AD ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT03706885), NCT03706885). The mechanisms of efavirenz effect on behavioral improvements in the 1st (amyloid-decreasing) and 2nd (amyloid-unchanging) paradigms are currently under investigation and will be published elsewhere. If briefly, efavirenz treatment seems to target microglia and lipid rafts as well as significantly

reduce protein phosphorylation. Other processes (plasma-dependent amyloid clearance, inflammation, oxidative stress, cellular hypoxia, autophagy, ubiquitin-proteasome systems, and apoptosis) could be affected as well, in part via changes in the Ca^{2+} -, small GTPase-, and catenin-signaling.

Besides in 5XFAD mice, efavirenz was tested in other experimental settings. Under the conditions of depression, the treatment of Swiss albino mice with the CYP46A1-activating efavirenz dose of 0.1 mg/kg body for 2 weeks decreased the immobility time in the forced swim test and increased sucrose intake as well as the brain serotonin levels. These results indicate a possible antidepressant efavirenz effect [113]. The CYP46A-inhibiting efavirenz dose of 15 mg/kg body weight was given with chow for 10 days to 8-month-old Tg2576 mice producing amyloid β . These mice had increases in the soluble amyloid β production and expression of β -secretase along with a reduction in microglial amyloid β phagocytosis as indicated by the cell culture studies [117]. Lastly, efficacy and toxicity screening in induced pluripotent stem cell-derived astrocytes and neurons showed that CYP46A1 activation by efavirenz reduces phosphorylated tau in early human AD neurons without affecting astrocyte viability [129]. Thus, evidence accumulates that efavirenz-induced CYP46A1 activation is generally beneficial, whereas enzyme inhibition could be deleterious in the context of AD.

CYP46A1 as a Target in Clinical Trials

Currently, CYP46A1 is investigated as a pharmacologic target in two clinical trials registered at [ClinicalTrials.gov](https://clinicaltrials.gov/): NCT03706885 and NCT03650452. As of the time of this review writing, both trials are in the patient recruitment phase. In the NCT03706885 trial, CYP46A1 will be evaluated for activation by the 50 mg/day or 200 mg/day efavirenz dose that will be given to stable patients with mild cognitive impairment due to AD. This is a proof-of-concept, two-center, randomized, double-blind, placebo-controlled clinical research study with the anticipated enrollment of 36 participants. Efavirenz will be evaluated for safety and tolerability in the geriatric population as well as the effect on CYP46A1 activity in the brain as indicated by changes in the serum 24HC levels (the primary endpoints). The secondary endpoint is a unique stable isotope kinetics labeling study, in which some patients will be given deuterated water to precisely measure their CYP46A1 activation in the brain and brain cholesterol turnover. In addition, all participants will be genotyped for the APOE isoform status ($\epsilon 2$, $\epsilon 3$, or $\epsilon 4$) and presence of specific SNPs in CYP46A1 and CYP2B6 (the major efavirenz-metabolizing enzyme). A post hoc

analysis will then be conducted to investigate whether these genetic factors affect a study participant's response to efavirenz treatment (the tertiary endpoint). Neurocognitive assessments and the measurements of amyloid β , total tau, and phospho-tau in the patient CSF will be conducted as well but are not among the trial endpoints.

In the NCT03650452 trial, CYP46A1 will be evaluated for inhibition by the drug called TAK-935 (OV935) developed by the Takeda Pharmaceutical Company. This is a phase 2, multicenter, randomized, double-blind, placebo-controlled study with the anticipated enrollment of 126 participants. TAK-935 (OV935) will be given as an adjunctive therapy to pediatric patients with Dravet syndrome (also known as severe myoclonic epilepsy of infancy) or Lennox–Gastaut syndrome (a severe form of epilepsy that typically becomes apparent during infancy or early childhood). TAK-935 (OV935) will be assessed for the efficacy, safety, and tolerability as compared to placebo. The primary endpoint of this trial is a percent change from baseline in frequency of all seizures (convulsive and drop) per 28 days on TAK-935 (OV935) therapy as compared to placebo. The secondary endpoints are multiple and include the correlation of the TAK-935 concentration and plasma 24HC levels. Thus, clinical data will ultimately become available on the effects of CYP46A1 activation and inhibition in different pathologic conditions.

Conclusions

There have been a tremendous increase in our knowledge of CYP46A1 since the finding in 1996 that the flux of 24HC from the brain is important for cholesterol homeostasis in the CNS [20] and subsequent cloning of *Cyp46a1* in 1999 [17]. Nevertheless, the significance of this enzyme for brain functions and potential as a therapeutic target for diseases of the brain are not yet fully understood and require further intensive investigations. These investigations should be conducted *in vivo* and with the appreciation that CYP46A1 activity can affect the brain cholesterol and 24HC levels as well as the rate of brain cholesterol turnover. In some conditions, these CYP46A1 effects will likely be uncoupled, thus enabling studies of the individual contributions of the CYP46A1 effects. We envision that the use of unbiased omics approaches on well-characterized samples of the whole brain, specific brain regions, and cell types will provide major advances in our understanding of the CYP46A1 role in health and disease conditions. Further development of genetic and pharmacologic tools that target CYP46A1 in specific brain regions or cell types will be of immense clinical and fundamental importance as well. Finally, there should be more clinical trials testing CYP46A1 as a therapeutic target for different brain diseases and conditions.

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