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REVIEW ARTICLE

Cholesterol in Brain Development and Perinatal Brain Injury: More than a Building Block

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Abstract: The central nervous system (CNS) is enriched with important classes of lipids, in which cholesterol is known to make up a major portion of myelin sheaths, besides being a structural and functional unit of CNS cell membranes. Unlike in the adult brain, where the cholesterol pool is relatively stable, cholesterol is synthesized and accumulated at the highest rate in the developing brain to meet the needs of rapid brain growth at this stage, which is also a critical period for neuroplasticity. In addition to its biophysical role in membrane organization, cholesterol is crucial for brain development due to its involvement in brain patterning, myelination, neuronal differentiation, and synaptogenesis. Thus any injuries to the immature brain that affect cholesterol homeostasis may have long-term adverse neurological consequences. In this review, we describe the unique features of brain cholesterol biosynthesis and metabolism, cholesterol trafficking between different cell types, and highlight cholesterol-dependent biological processes during brain maturation. We also discuss the association of impaired cholesterol homeostasis with several forms of perinatal brain disorders in term and preterm newborns, including hypoxic-ischemic encephalopathy. Strategies targeting the cholesterol pathways may open new avenues for the diagnosis and treatment of developmental brain injury.

Keywords: Cholesterol, brain development, brain injury, CNS, encephalopathy, neuroplasticity.

1. INTRODUCTION

Cholesterol is commonly known as an integral component of the cell membrane to maintain membrane stability and fluidity, and regulate transmembrane protein signaling as a major organizer of lipid rafts. Cholesterol is also deployed in the synthesis of lipoproteins, steroid hormones such as progesterone, Vitamin D, and bile salts. While most peripheral organs use exogenous cholesterol produced mainly in the liver and transported in the bloodstream in the form of low-density lipoprotein (LDL) and very-low-density lipoproteins (VLDL), the brain has to rely on local synthesis once the blood-brain barrier (BBB) is fully developed and impermeable to circulating cholesterol that is carried in lipoproteins. In fact, the brain is the most cholesterol-rich organ in the body. The human brain has an average cholesterol concentration of 7 mg/g at birth, which is in contrast to 1.5 mg/g in other organs [1]. The human brain contains nearly 25% of the body's entire amount of cholesterol, with the majority (up to 70-80%) present in myelin sheaths that aid in rapid saltatory nerve conduction, and the rest in the membranes of neurons, glia cells, and other cellular elements [2]. Brain cholesterol removal is also unique in its hydroxylation by the brain-specific cholesterol 24-hydroxylase (also known as CYP46A1) to generate 24S-hydroxycholesterol (24S-HC), which is able to pass the BBB to the circulation, where it is excreted by the liver.

Normal brain function requires balanced cholesterol homeostasis, especially during the phase of maximal membrane growth and myelinogenesis in the developing stages. Unfortunately, there is a dearth of information regarding the dynamics and regulation of cholesterol production, transportation, distribution, utilization, and removal in the immature brain, as well as how these processes relate to brain function and shape neuroplasticity during the critical period. Moreover, few data are available in the literature on cholesterol dysregulation in the context of common types of grey and white matter injuries in full-term and preterm infants, including hypoxic and/or ischemic brain damage. This review will discuss the above topics using the findings from the current rodent preclinical and human studies in a pediatric population.

2. SOURCES OF CHOLESTEROL IN FETAL AND NEWBORN BRAIN

Cholesterol provides important life support for embryonic and fetal development. Mouse studies found that in early gestation, the fetus, including the fetal brain, obtains exogenous cholesterol transported from maternal circulation through the yolk sac and placenta [1, 3-5]. Primarily, LDL- or HDL-cholesterol is taken up by the fetus via LDL receptor or scavenger receptor class B type I (SRB1, in humans, also

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termed CLA-1) respectively, which are expressed in trophoblasts and the endothelium of the fetoplacental vasculature [4, 5]. ATP-binding cassette (ABC)-transporters, ABCA1 and ABCG1, are also involved in cholesterol excretion from the two cell layers separating the maternal and fetal circulation [3, 5]. Starting E13-14, the endogenous synthesis in the liver and lung becomes increasingly robust to accommodate rapid tissue growth [6]. Maternal-fetal cholesterol transport is effective in other species, too, especially during early development, including humans. The human fetus depends on the maternal cholesterol supply until at least 19 weeks of gestation [7]. There is a significant correlation of plasma cholesterol levels between the mothers and the fetuses that are younger than 6 months, but not at an older age when the fetus produces the majority of cholesterol by itself [8]. Evidence also shows that fetuses with intrauterine growth restriction (IUGR) delivered between 6 months and full-term have cholesterol deficiency compared to normally grown fetuses due to reduced de novo synthesis rather than diminished maternal supply [9].

As for brain cholesterol, since the circulating cholesterol carried in blood lipoproteins cannot cross the BBB, after BBB establishment around E10-11 in mice, the fetal brain has to make almost all of its own cholesterol, *i.e.*, 85-90%, from E15 to birth [6]. It is unclear whether the human BBB is fully developed and operative before or after birth because some of its components continue to mature after birth. As such, the specific timing of BBB closure to blood cholesterol, the relative contribution, and the importance of brain cholesterol derived from the peripheral circulation or made locally during development has never been demonstrated in humans.

3. CHOLESTEROL BIOSYNTHESIS AND ACCRETION DURING BRAIN DEVELOPMENT

As noted, brain cholesterol depends on de novo synthesis by local brain cells, rather than taking up the circulating cholesterol-containing lipoprotein as the cells of other parts of the body. A mouse quantitative study reported that the rate of cholesterol synthesis in the CNS in the first 3 weeks is approximately 0.28 mg/day, and almost all cholesterol is accumulated for membrane expansion and active myelination (at a rate of 0.26 mg/day) [10]. These rates highly correlate with the rates of synthesis of myelin basic protein (MBP) and cerebroside, two other important myelin components [11]. These rates are 10 times higher than those at adult age (between 13 and 26 weeks) when the cholesterol pool is relatively inert. There are no gender differences in the total amount or the rate of cholesterol synthesis in the CNS in 7-week-old mice [10].

The pathway of brain cholesterol synthesis is similar to that in the liver, which involves multiple enzymatic reactions and intermediates with Acetyl-CoA as the starting material and HMG-CoA reductase (HMGCR) as the overall rate-limiting enzyme (Fig. **1**). This is an energetically expensive process, during which production of 1 mole of cholesterol requires 18 moles of acetyl-CoA, 36 moles of ATP and 16 moles of NADPH. Eleven oxygen molecules are needed along the pathway, 10 of which are consumed for the conversion of lanosterol to cholesterol that can follow either of the so-called Bloch or Kandutsch-Russell Pathway. The two pathways utilize essentially the same enzymatic machinery with desmosterol, and 7-dehydrocholesterol as the immediate final precursor for cholesterol, respectively. It is unique to the developing brain, but not to other tissues, that desmosterol increases prenatally and peaks in the first postnatal week in mice [10, 12, 13] or rat [14], which at that time represents 30% of the total brain sterol [10]. Desmosterol diminishes quickly and disappears by 20 days in rodents [10, 12, 13, 15]. This is accompanied by the accelerating cholesterol synthesis and myelination over the same period. In the human fetal brain, it has been suggested that desmosterol accumulates between 10 to 20 weeks [16] and is in trace amounts at birth and absent in the adult brain. These *data* support the notion that an earlier life deposition of desmosterol and rapid conversion of desmosterol to cholesterol precede the onset of myelination. The mechanisms and physiological significance of this transient desmosterol accumulation are unclear. An *in vitro* study speculated that it may be caused by progesterone, or related steroids, that inhibit the activity of 24-dehydrocholesterol reductase (DHCR24), which converts desmosterol to cholesterol [12]. However, whether this is the case *in vivo* in the developing brain is unknown. Desmosterol may have additional roles in neuronal arborization and synaptic plasticity in a particular time window of brain development [13].

Similar to desmosterol, other upstream cholesterol precursors are biologically active with important physiological functions. Lanosterol, the first sterol committed to cholesterol synthesis and, therefore, considered as a surrogate for cholesterol, is able to induce mild depolarization of mitochondria and promote autophagy and protect dopaminergic neurons from cell death in a model of Parkinson's disease [17]. Higher plasma lathosterol, another marker of endogenous cholesterol synthesis, is associated with impaired white matter development and worse motor outcomes in preterm newborns [18]. Further upstream in the isoprenoid pathway, geranylgeraniol (GG) formed from farnesyl pyrophosphate (FPP) and geranylgeranyl-PP is found to be required for hippocampal long-term potentiation (LTP) and learning/memory [19]. These *data* highlight the necessity of examining cholesterol synthetic intermediates, not only the end product cholesterol, in conditions with aberrant cholesterol homeostasis. Moreover, as a change in a single enzyme or intermediate can set off a chain reaction affecting other molecules along the pathways, it is challenging to determine the root cause or initial driver of all changes for identifying appropriate drug targets.

At the transcriptional level, cholesterol biosynthesis is regulated by the sterol regulatory element-binding protein (SREBP) family of transcription factors, preferentially SREBP2 [20, 21]. SREBP-1a and SREBP2 activate a range of enzymes in the cholesterol synthetic pathway, including HMGCS, HMGCR, FPP synthase (FPPS), squalene synthase (SQS), Cyp51A1, DHCR7, *etc.* [22]. SREBP transcription is mainly controlled by intracellular sterol levels.

4. THE CONTRIBUTION OF INDIVIDUAL CELL TYPES TO THE CHOLESTEROL POOL IN THE DEVELOPING BRAIN

The common belief among neuroscientists that the neurons rely on cholesterol transported from astrocytes stems

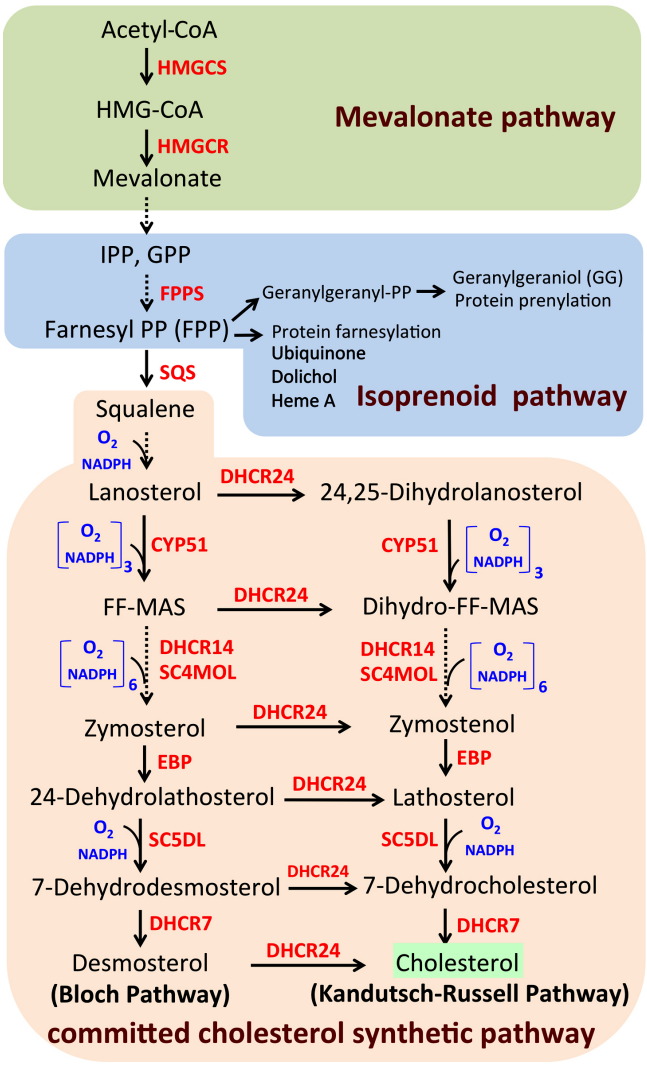

Fig. (1). Cholesterol biosynthetic pathway: Cholesterol synthesis uses Acetyl-CoA as starting material and is illustrated here as pre-squalene mevalonate pathway and post-squalene pathway that is committed to cholesterol biosynthesis. The isoprenoid pathway utilizes isoprenoids (mainly FPP and geranylgeranyl-PP) for protein farnesylation and prenylation. HMG-CoA: 3-hydroxy-3-methylglutaryl-*CoA,* IPP: isopentenyl pyrophosphate, GPP: geranyl pyrophosphate, HMGCS: HMG-CoA synthase, HMGCR: HMG-CoA reductase, FPPS: farnesyl pyrophosphate synthase, SQS: squalene synthase, DHCR24: 24-dehydrocholesterol reductase, CYP51: Cytochrome P450 family 51 subfamily, DHCR14: 14-dehydrocholesterol reductase, SC4MOL: sterol-C4-methyl oxidase-like, EBP: emopamil binding protein, SC5DL: sterol C5-desaturase-like, DHCR7: 7-dehydrocholesterol reductase. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

from the studies in adult rodents or primary cultures with uncertain maturation stages. The purpose of this outsourcing is to save energy for neuronal activity. Indeed, when endogenous cholesterol synthesis is abolished genetically in neurons in the postnatal or adult mouse brain, they can survive and function, probably by utilizing cholesterol derived from astrocytes [23, 24]. However, there are critical time windows during brain development when neurons produce cholesterol prolifically and indispensably for neurite outgrowth. Mouse embryonic subcortical projection neurons lacking endogenous cholesterol production either die or have morphological deficits (shorter projections), which cannot be rescued by glial support [23]. *In vitro data* from separate cortical neuronal and astrocyte cultures revealed that developing neurons (3-9 days *in vitro*) produce significantly more cholesterol per cell than astrocytes, while astrocytes release more cholesterol into the medium than neurons [25]. These findings indicate that neurons are the main cholesterol producers in the grey matter during CNS development to serve their own membrane growth. In the intact brain, a horizontal cholesterol transportation system between neurons, astrocytes, and microglia has been proposed [26]. Whether this system exists in the developing brain or how their integration is tightly regulated, how the cells sense and respond to the changes in cholesterol amount in their neighbor cells or environment remain unknown.

The prolonged myelination process begins prenatally in humans, but primarily postnatally in rodents, during which most of the cholesterol synthesis is taking place in oligodendrocytes (OLGs). Oligodendroglia cholesterol synthesis is rate limiting for myelination in the white matter [27]. Meanwhile, astrocytes also provide a substantial amount of choles-

terol that is incorporated into myelin during normal brain development [28].

5. BRAIN CHOLESTEROL METABOLISM IN THE DEVELOPING BRAIN

The major pathway of brain cholesterol elimination is its hydroxylation at the C24 position to generate oxysterol 24*S*-hydroxycholesterol (24S-HC). Oxysterols are oxygenated cholesterol metabolites with hydroxyl or epoxy groups in their isooctyl side chains. Unlike cholesterol, 24S-HC can readily cross the BBB into the circulation to the liver for excretion [29]. This pathway controls cholesterol removal and fluxes out of the brain to maintain its cerebral balance. The enzyme that mediates this conversion, CYP46A1 (in humans, Cyp46a1 in mice), is a unique member of the cytochrome P450 family as it is primarily expressed in the brain and by neurons [30, 31]. Studies with Cyp46a1 knockout mice suggest that this enzyme is responsible for the production of almost all (≈ 98-99%) of the 24S-HC in the brain and ≈ 60-80% 24S-HC in the serum [32]. Most circulating 24S-HC has a cerebral origin [33], and thus the serum level of 24S-HC could be an indication of cholesterol metabolism in the brain [34]. This pathway also controls brain cholesterol neogenesis, as de novo cholesterol synthesis was reduced by 40% in the Cyp46a1 knockout mice, while the total cholesterol amount was unchanged when analyzed at 7 weeks of age [32].

The spatial and temporal expression of Cyp46a1 demonstrated that in postnatal day 9 (P9) mouse brain, it is localized in the large pyramidal neurons in cortical layers V and VI, hippocampus, striatum and thalamus (Fig. **2**), namely the most metabolically active neurons [35]. This is similar to its distribution in the adult mouse brain [30, 36]. In humans and mice, brain CYP46A1 protein expression is low at birth but increases during development and reaches a peak ≈ 3 years in humans or 2-4 weeks in mice and remains steady through adulthood. Accordingly, the blood concentration of 24S-HC in mice increases by 2 weeks and then declines with age [36-38]. It is not surprising that the trajectory of Cyp46a1 resonates with the timing of myelination or neuronal development as most cholesterol is deposited at an early stage. Once a stable pool is built up as the animal matures, Cyp46a1 is increased to eliminate surplus cholesterol from the brain, thereby maintaining cholesterol homeostasis for normal brain function.

6. ROLE OF CHOLESTEROL IN FETAL AND POSTNATAL BRAIN DEVELOPMENT

It is well recognized that cholesterol plays a highly specialized role in the developing brain. It participates in multiple key processes of brain development, from the earliest brain patterning to neuronal differentiation, synaptogenesis, and myelination. In this section, we will discuss these pivotal roles in detail.

6.1. Cholesterol is Indispensable for Patterning of the Fetal Nervous System

The best example of the involvement of cholesterol in CNS development is its modification of the Hedgehog (Hh) signaling pathway. Cholesterol is known to be an inducer of the Hh family of morphogens, which play myriad roles in directing proper cell differentiation and specifying cell fates/regions in the early development of various tissues, especially CNS, limbs, skeleton, skin, lung, *etc.* [39]. There is a defective response to Hh signaling in disorders of cholesterol biosynthesis [40]. Cholesterol is required for both Hh precursor processing in Hh-generating cells and signal reception in Hh-responding cells to coordinate intercellular communication. In the sonic hedgehog (SHH) signaling pathway, cholesterol is covalently attached to the carboxyl terminus of SHH protein following its autocatalytic cleavage [41], along with other lipid modifications, leading to SHH activation and stabilization. The protein is then secreted, transported to distant responding cells, and binds to the receptor Patched 1 (PTCH1) on the surface of the target cells. As another level of regulation on the receiving cells, it has been recently proposed that cholesterol, more accurately accessible cholesterol on the membrane, serves as a second messenger and conveys the signal from PTCH1 to Smoothened (SMO), which is activated and further transmits/propagates SHH signal to intracellular/nuclear components [42-45].

Very early in gestation, SHH is involved in neural patterning and the development of the forebrain, as well as cerebellum [46], aside from the limbs and heart [39]. Cholesterol modification of SHH is essential for dorsoventral patterning in the telencephalon [47]. Mutations in the human SHH gene cause holoprosencephaly [48, 49] and other major congenital disorders. In addition, SHH proteins play a role in the migration and survival of neural crest cells, a population of cells that highly contribute to the development of the brain, limbs, lungs, heart and urogenital system [50, 51]. Hh signal transduction between astrocytes and BBB endothelial cells promotes BBB formation and maturity during embryonic development [52]. This interaction restricts the permeability of the BBB, limiting the extravasation of blood-derived inflammatory molecules into the CNS [52]. In the adult brain, SHH is required for the maintenance and migration of adult neural stem cells in the subventricular zone and the hippocampal subgranular zone [53], which are implicated in repair in response to brain injury.

6.2. Cholesterol Facilitates Synaptogenesis and Plasticity

The involvement of cholesterol in synapse formation and activity was initially reported in 2001 [54]. This elegant *in vitro* study found that glia-derived cholesterol carried in apoE-containing lipoproteins increased the number and efficacy of synapses in rat retinal ganglion cells (RGC), while the endogenous cholesterol made by RGC can only support survival and produce a few immature synapses [54, 55]. The subsequent experiments identified dendrite differentiation as rate limiting for glia-induced synaptogenesis, which requires cholesterol [56]. Additionally, glia-derived lipoproteins stimulate RGC axon growth, which was diminished by blocking cholesterol synthesis in astrocytes [57]. Inhibition of endogenous cholesterol synthesis in pure rat cortical neuronal cultures also interfered with dendrite outgrowth due to decreased microtubule stability as a result of dephosphorylation of microtubule-associated protein 2 (MAP2) [58]. Furthermore, cholesterol deficiency caused tau hyperphosphorylation and microtubule depolymerization in axons in neuronal cultures [59]. These findings point to multiple roles for

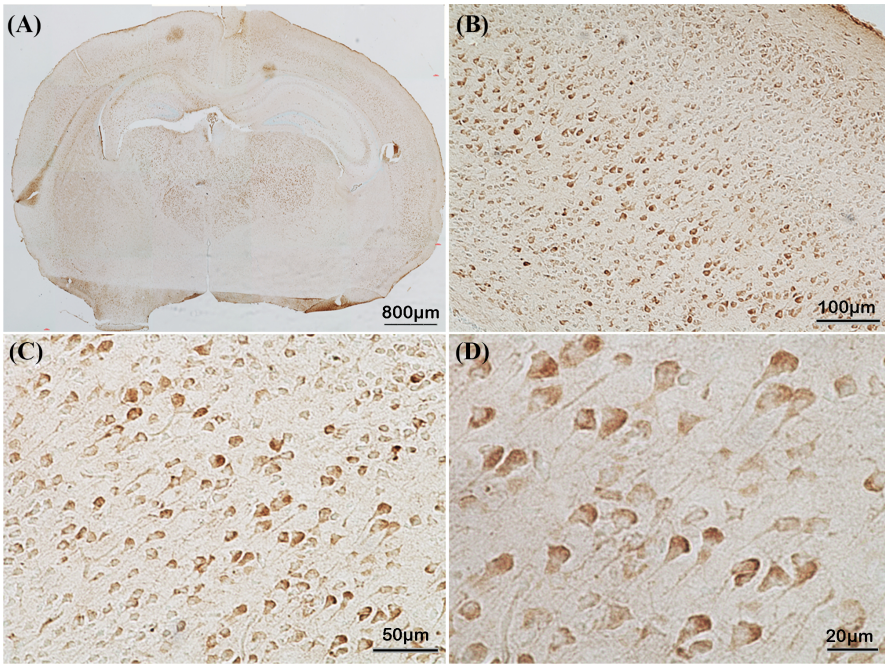

Fig. (2). Expression of Cyp46a1 in P9 mouse brain: Immunohistochemistry staining of P9 naïve mouse brain with anti-Cyp46a1 antibody shows its expression in neurons in the cortex, hippocampus, striatum and thalamus (**A**). In the cortex, Cyp46a1 is primarily localized in the large pyramidal neurons in layers V, VI, and layers II, III (**B**). Higher magnification demonstrates its strong signal in the soma and dendrites (**C, D**). The 12 μm mouse brain cryo-sections were used for the staining with mouse anti-Cyp46a1 antibody (1:100, MAB2259, MilliporeSigma). *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

cholesterol in regulating neuronal differentiation, including axon and dendrite outgrowth, that are prerequisites for synaptogenesis. For the synaptic elements, cholesterol plays a crucial role at both presynaptic sites (facilitating vesicle formation and transport, as well as transmitter release) and postsynaptic terminals (enhancing postsynaptic receptor clustering [60] and organizing neurotransmitter receptors and their associated proteins as a major component of membrane lipid rafts) [56, 61]. Moreover, cholesterol is required for continued synapse development [56]. Cholesterol depletion leads to synaptic and dendritic spine degeneration, failed neurotransmission, and decreased synaptic plasticity [62]. A more specific modulatory function of cholesterol on synaptic transmission was uncovered recently, showing that cholesterol controls the open probability and function of the NMDA-type glutamate receptor and stabilizes these receptors in synapses in cultured rat cerebellar granule cells and hippocampal neurons [63, 64].

It is reasonable that the level of synaptic cholesterol, but not the total amount of intracellular cholesterol, is responsible for its modulation of synaptic function. Overexpression of CYP46A1 led to a lower content of synaptosomal cholesterol with increased dendritic outgrowth and synaptic markers [65]. This seems to be contradictory to the above findings. The results from both CYP46A1 transgenic and Cyp46a1 knockout mice show that manipulating brain cholesterol turnover modulates cholesterol synthesis with altered activity of upstream mevalonate pathway, which in turn affects the relevant side reactions of protein prenylation and the activity of small GTPases that are associated with cholesterol-mediated effects on dendritic differentiation and synapse function [19, 65].

Most of the findings concerning cholesterol modulation of synapse formation and function are derived from *in vitro* culture systems using cholesterol depletion reagents or inhibitors of cholesterol biosynthesis. Whether the availability of cholesterol in the brain determines the extent of synaptogenesis during development or whether external cholesterol from astrocytes is needed in this process, or if cholesterol is required for the formation of all types of synapses, and whether disturbance of cholesterol production or transportation has an effect on synaptic plasticity *in vivo*, are all unknown.

6.3. Cholesterol is Essential for Oligodendrocyte Differentiation and Myelination

The majority of brain cholesterol (70%) is stored in myelin sheaths in the white matter, and around 40% of lipid components of myelin is cholesterol (more than any other single lipid) [66], highlighting the importance of cholesterol in myelination. In myelin, cholesterol inserts into the membrane bilayers to increase myelin viscosity and stabilize myelin lipids and proteins [67]. High cholesterol levels are essential for myelin membrane growth, and cholesterol availability in OLGs is a rate-limiting factor for brain maturation [27]. During postnatal myelination, myelin cholesterol is made locally by OLGs [68], and in addition, supplemented by neighboring astrocytes [28] through ApoE-containing lipoproteins. Mice lacking cholesterol synthesis specifically in OLGs showed hypomyelination with deficits in motor function, such as ataxia and tremors. The mutant OLGs can survive and mature probably by taking cholesterol from surrounding wild-type cells [27].

On the other hand, cholesterol is important for OLG differentiation. Isoprenoids and protein prenylation are required for OLG migration arrest, while cholesterol is necessary for axon ensheathment and myelin gene expression [69, 70]. Blocking SREBP processing and thus cholesterol synthesis in OLGs inhibited OLG differentiation and expression of MBP in cultured cortical OLGs [71]. Likewise, statin treatment increased the number of OLG precursors and decreased the number of mature OLGs, and caused retraction of OLG processes [72], suggesting a role of cholesterol in OLG maturation.

7. CHOLESTEROL DYSREGULATION IN NEONATAL HYPOXIC-ISCHEMIC BRAIN INJURY

Mutations in genes encoding the enzymes that are involved in the cholesterol biosynthetic pathway or cholesterol trafficking/transport are linked to genetic disorders with significant malformations and neurodevelopmental delay. The related human diseases and animal models have been summarized in considerable detail elsewhere [73-75]. Here, we discuss cholesterol dysregulation in acquired perinatal brain injury, for example, hypoxic-ischemic encephalopathy (HIE). HIE caused by hypoxia and/or reduced cerebral blood flow is a severe birth complication affecting full- or near full-term newborns. Occurring in 3 per 1000 live births, HIE is a leading cause of perinatal death and lifelong neurological morbidity [76, 77]. Studies on the pathophysiology of hypoxic-ischemic (HI) brain damage have been focused largely on protein-associated signaling pathways. Current knowledge on how the lipid components, including cholesterol, are affected and involved in the injury or repair processes is lacking. As an oxygen-consuming process that requires 11 oxygen molecules for the conversion of Acetyl-CoA to cholesterol, cholesterol synthesis is thus sensitive to reduced O_2 availability. This is true in the Vannucci model of neonatal HI, as reported by two earlier studies, showing sustained loss of brain cholesterol during the first 3 days in the ipsilateral hemisphere [78] or 7 days to 3 months in the hippocampus [79] after HI in P7 rats. We also observed reduced brain cholesterol in the ipsilateral cortex at 6 hour after HI in P9 mice [80]. These *data* suggest a possible disruption of brain cholesterol synthesis following HI. A more recent transcriptome analysis reported a transient inhibition of 12 genes encoding cholesterol synthetic enzymes at 12 hour after HI at P5, but not in P10 mice after HI [81], indicating an age-dependent response and a more profound impact at an earlier age that may be implicated in myelin and white matter injury. The direct and long-term consequences of altered cholesterol synthesis after neonatal HI need further investigation.

Cholesterol loss after neonatal HI could be a result of increased cholesterol turnover as well. We showed activation of Cyp46a1 at 6 hour and 24 hour post-HI with a concomitant increase in the brain and serum levels of 24S-HC [80]. The correlation between the serum and brain levels of 24S-HC corroborates the brain origin of circulating 24S-HC. The findings that the serum levels of 24S-HC at 6 hour and 24 hour correlate with grey and white matter injury, as well as with long-term motor and cognitive function, suggest that 24S-HC could be a novel and early blood biomarker for the severity of neonatal HI brain damage and associated functional impairments [35]. This could aid in the identification or severity stratification of HIE brain injury in the clinical setting. In fact, this oxysterol was demonstrated as a serum biomarker for disease activity and progression in multiple neurodegenerative disorders [82, 83]. Children with autism spectrum disorders have a significantly higher level of plasma 24S-HC [84], while infants with Smith-Lemli-Opitz syndrome have reduced concentrations [85]. How exactly and why disruption of cholesterol metabolism is associated with these diseases are unclear. In the setting of neonatal HI, Cyp46a1 can be activated by oxidative stress [38, 86] or glutamate [87]; both are associated with cell death, and with the activation of the NMDA receptors (NMDAR), the major glutamate receptors mediating excitotoxicity in neonatal brain injury. 24S-HC is, in turn, an endogenous positive allosteric modulator of the NMDAR (preferentially the GluN2B-containing NMDAR) to enhance excitatory neurotransmission [88-90]. In an *in vitro* model of HI (oxygen-glucose deprivation, OGD) in hippocampal neurons, overexpression of Cyp46a1 increased, whereas knockout of Cyp46a1 attenuated OGD-induced cell death via NMDAR activation [91]. These *data* support the role of Cyp46a1/24S-HC in mediating neuronal death. However, another possibility cannot be excluded that 24S-HC may be released from dying neurons or during acute demyelination following HI when excess free cholesterol from disrupted plasma membranes or myelin breakdown at the site of injury needs to be cleared out by hydroxylation to 24S-HC. In this view, 24S-HC is a byproduct of cell death. More studies are needed to better understand the causal relationship between brain damage and Cyp46a1 activation in neonatal hypoxia-ischemia.

Another example of cholesterol modulating ischemic neuronal injury was that activation of SREBP1 is required in NMDAR-induced neuronal death in both *in vitro* (using day 12-14 cortical neurons) and *in vivo* models of stroke in adult rats [92]. The transcriptional factor SREBP1 regulates lipid biosynthesis, including cholesterol. Treatment of primary cortical neurons with a high level of cholesterol prevented NMDA-mediated SREBP1 activation and reduced excitotoxicity [92]. This is consistent with the finding that excitotoxicity causes cholesterol loss [93], which could lead to SREBP1 activation and neuronal death. Knockdown of Cyp46a1 inhibited glutamate-mediated cholesterol loss [93], which might be neuroprotective.

8. PLASMA CHOLESTEROL AND BRAIN DEVELOPMENT IN PRETERM NEWBORNS

As mentioned above, maternal cholesterol is critical for fetal growth, including brain development. Studies have shown that either high [94, 95], or low [96] levels of maternal cholesterol increase the risk for premature birth. In addition, a clinical study assessing the relationship between plasma cholesterol levels and brain development in 60 preterm infants showed that higher early endogenous cholesterol synthesis represented by higher lathosterol levels is associated with subcortical white matter edema and worse motor outcomes. Higher early blood cholesterol is associated with improved cerebellar volumes [18]. Although plasma cholesterol is separate from brain cholesterol, the issues in nutrition/metabolism, infections/immune system, the liver, or cardiovascular system that affect blood cholesterol may

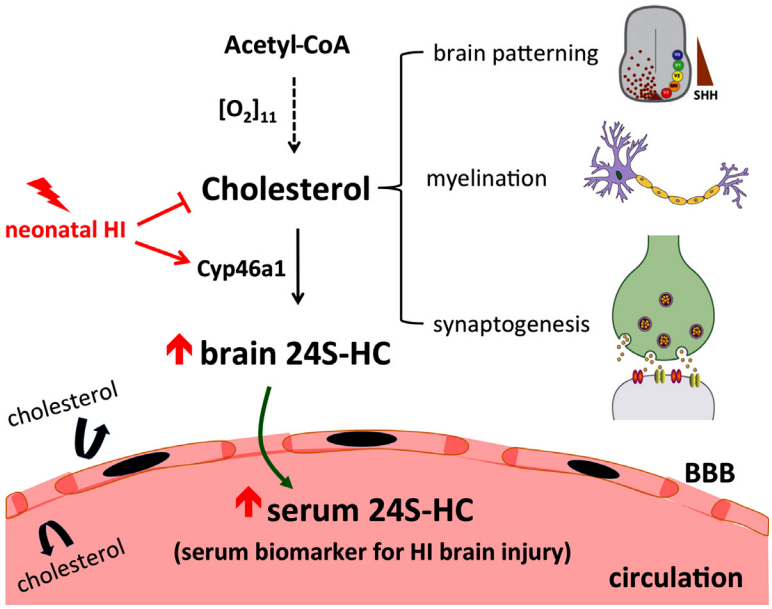

Fig. (3). Cholesterol in brain development and neonatal brain hypoxia-ischemia (HI): Brain cholesterol relies on de novo biosynthesis from Acetyl-CoA and is removed from the brain by hydroxylation with Cyp46a1 into 24S-HC, which is able to cross BBB into the circulation. Cholesterol plays critical roles in brain development by regulating brain patterning, myelination and synaptogenesis. Neonatal HI leads to brain cholesterol loss by inhibiting cholesterol synthesis and activating cholesterol metabolism with increased production of 24S-HC in the brain and serum. The serum level of 24S-HC might be a novel lipid-based biomarker for the severity of HI brain injury. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

indirectly impact brain development in premature babies. In the cases when BBB integrity is compromised, plasma cholesterol may penetrate and contribute to the brain cholesterol pool.

9. LIPID RAFTS LINK CHOLESTEROL DYSFUNCTION TO NEURODEVELOPMENTAL DISORDERS

Lipid rafts are dynamic membrane microdomains rich in cholesterol and sphingolipids and are important platforms for transmembrane protein signal transduction [97-99]. Cholesterol serves as a spacer between the hydrocarbon chains of the sphingolipids and functions as dynamic glue that keeps the raft assembly together. Removal of raft cholesterol leads to raft dispersion, dissociation of most proteins from rafts and renders them nonfunctional [100]. Therefore, cholesterol depletion reagents, such as methyl-β-cyclodextrin (Mβ-CD) are commonly used to study the function of lipid rafts and raft proteins in physiological or disease conditions. One example of lipid raft regulating proteins that are implicated in neurodevelopmental disorders is the L1 cell adhesion molecule (L1). The role of L1 in promoting neurite outgrowth is dependent on lipid rafts. L1 function in the lipid rafts is disrupted by ethanol, which is associated with fetal alcohol syndrome (FAS) with deficits in brain morphology and mental retardation [101]. The same mechanisms underlie the neurotoxicity of toluene, leading to neurodevelopmental disabilities similar to FAS [102]. Likewise, bilirubin in hyperbilirubinemia, which is associated with cerebellar dysfunction and dystonia in full-term and premature newborns, exerts neurotoxic effects by inhibiting lipid raft-dependent L1 function in rat cerebellar granule neurons [103]. Lipid rafts also link cholesterol metabolism to synaptic deficits in autism spectrum disorders [104], including Rett syndrome [105].

10. TARGETING CHOLESTEROL PATHWAYS FOR THE NOVEL INTERVENTION OF PERINATAL BRAIN INJURY

Studying cholesterol homeostasis disturbance and the associated consequences not only helps us understand its contribution to the pathogenesis of perinatal brain injuries but also facilitates the identification of potential druggable targets on the cholesterol pathways to ameliorate brain injury or promote regeneration in infants. There are not many preclinical studies with treatment strategies on the axis of cholesterol pathways, since it is an under-recognized mechanism compared to other protein pathways.

10.1. Cholesterol-lowering Drugs

Animal studies and clinical trials have proven that statins, the most widely used cholesterol-lowering drugs, are neuroprotective in adult stroke. Statins inhibit HMGCR, the rate-limiting enzyme in the cholesterol biosynthetic pathway. It is surprising that the underlying mechanisms are unrelated to its cholesterol-reducing action but rather to its role in activating endothelial nitric oxide synthase (eNOS), which regulates vascular tone and improves perfusion as well as its anti-inflammation or anti-oxidative effects. These effects are associated with reduced production of isoprenoids, such as farnesyl-PP and geranylgeranyl-PP, that regulate small GTP-binding proteins [106, 107]. In the rat model of neonatal brain hypoxia-ischemia, prophylactic but not delayed administration of simvastatin improved long-term morphological and behavioral outcomes [108] by attenuating inflammatory

responses [109], but not eNOS expression [110] or through the activation of protein kinase B (Akt) and cAMP-response element-binding protein (CREB) [109]. Additionally, it protects against hypomyelination induced by HI in neonatal rats [111]. A recent study showed that simvastatin preconditioning may modulate autophagy and survival pathways by affecting mammalian targets of rapamycin (mTOR) C1, mTORC2, and the NAD^+-dependent protein deacetylase sirtuin 1 (SIRT1) activities in neonatal rats [112]. Despite these beneficial effects, the safety *data* for using simvastatin in newborn infants are lacking,

10.2. Cyp46a1 Inhibitor TAK-935

As noted, 24S-HC, the enzymatic product of Cyp46a1, enhances NMDAR-mediated excitatory neurotransmission. This action is also independent of its function in regulating cholesterol homeostasis. In the cases of NMDAR hyperactivity, for example, seizure and epilepsy, inhibition of Cyp46a1 and thereby reducing NMDAR overactivation could provide therapeutic benefits. TAK-935 (brand name: Soticlestat) is a novel, CNS permeable, highly selective, and potent competitive Cyp46a1 inhibitor developed by Takeda and Ovid Therapeutics Inc, currently in a clinical trial to treat pediatric patients with rare pediatric epilepsies, such as Dravet syndrome (severe myoclonic epilepsy of infancy) or Lennox-Gastaut syndrome (a severe form of epilepsy that becomes apparent during infancy or early childhood) [113-120]. Its tolerability in neonates and implication in perinatal brain injuries involving excitotoxicity need to be investigated. It would be interesting to test it in the mouse neonatal HI model in which Cyp46a1 is upregulated.

CONCLUSION AND PERSPECTIVES

In summary, cholesterol plays remarkably versatile roles in brain development. Beyond being a building block of cellular membranes, it has a multitude of functions, especially in brain patterning, neuronal differentiation, synapse formation and myelination (Fig. **3**). Through these processes, cholesterol coordinates intracellular, trans-membrane, and secretory signaling, and neuron-glia, neuron-endothelial cell interactions. Normal brain function requires balanced cholesterol homeostasis, especially during the phase of maximal membrane growth and myelinogenesis. Unfortunately, there is a dearth of information regarding brain cholesterol dysregulation in the critical period following injury. Future studies are needed to elucidate the region- and cell type-specific function of cholesterol in the developing brain and the long-term consequences of cholesterol dysregulation following perinatal brain injuries.

LIST OF ABBREVIATIONS

24S-HC	=	24S-hydroxycholesterol
ABCA1	=	ATP-binding Cassette sub-family A Member 1
ABCG1	=	ATP-binding Cassette Sub-family G Member 1
Akt	=	Protein kinase B
BBB	=	Blood Brain Barrier
CLA-1	=	CD36 and LIMPII Analogous-1
CNS	=	Central Nervous System
CREB	=	cAMP-response Element-binding Protein
CYP46A1	=	Cytochrome P450 Family 46 Subfamily A Member 1
CYP51A1	=	Cytochrome P450 Family 51 Subfamily A Member 1, Lanosterol-14α-demethylase
DHCR7	=	7-dehydrocholesterol Reductase
DHCR14	=	14-dehydrocholesterol Reductase
DHCR24	=	24-dehydrocholesterol Reductase
EBP	=	Emopamil Binding Protein
eNOS	=	Endothelial Nitric Oxide Synthase
FAS	=	Fetal Alcohol Syndrome
FF-MAS	=	Follicular Fluid Meiosis Activating Sterol
FPP	=	Farnesyl Pyrophosphate
FPPS	=	Farnesyl Pyrophosphate Synthase
GG	=	Geranylgeraniol
GPP	=	Geranyl Pyrophosphate
IPP	=	Isopentenyl Pyrophosphate
IUGR	=	Intrauterine Growth Restriction
Hh	=	Hedgehog
HI	=	Hypoxic-ischemic
HIE	=	Hypoxic-ischemic Encephalopathy
HMG-CoA	=	β-Hydroxy β-methylglutaryl-*CoA,* 3-hydroxy-3-methylglutaryl-*CoA*
HMGCR	=	HMG-CoA Reductase
HMGCS	=	HMG-CoA Synthase
LDL	=	Low-density Lipoprotein
LTP	=	Long-term Potentiation
MAP2	=	Microtubule Associated Protein 2
Mβ-CD	=	Methyl-β-cyclodextrin
MBP	=	Myelin Basic Protein
mTOR	=	Mammalian Target of Rapamycin
NMDAR	=	*N*-methyl-D-aspartate Receptor
OGD	=	Oxygen-glucose Deprivation
OLGs	=	Oligodendrocytes
PTCH1	=	Patched 1, Protein Patched Homolog 1
RGC	=	Retinal Ganglion Cells
SC4MOL	=	Sterol-C4-methyl Oxidase-like
SC5DL	=	Sterol C5-desaturase-like
SHH	=	Sonic Hedhehog
SIRT1	=	Sirtuin 1
SMO	=	Smoothened
SQS	=	Squalene Synthase

SRB1 = Scavenger Receptor Class B type 1

SREBP = Sterol Regulatory Element-binding Protein

VLDL = Very Low-density Lipoprotein

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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