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Oxysterols and the NeuroVascular Unit (NVU): a far true love with bright and dark sides

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Abstract:

The brain is isolated from the whole body by the blood-brain barrier (BBB) which is located in brain microvessel endothelial cells (ECs). Through physical and metabolic properties induced by brain pericytes, astrocytes and neurons (these cells and the ECs referred to as the neurovascular unit (NVU)), the BBB hardly restricts exchanges of molecules between the brain and the bloodstream. Among them, cholesterol exchanges between these two compartments are very limited and occur through the transport of LDLs across the BBB. Oxysterols (mainly 24S and 27-hydroxycholesterol) daily cross the BBB and regulate molecule/cholesterol exchanges via Liver X nuclear Receptors (LXRs). In addition, these oxysterols have been linked to pathological processes in neurodegenerative diseases such as Alzheimer's disease. Here we propose an overview of the actual knowledge concerning oxysterols and the NVU cells in physiological and in Alzheimer's disease.

Keywords: Blood-Brain Barrier, Neurovascular unit, oxysterols, cholesterol homeostasis, Alzheimer's disease

Conflict of interest:

The authors declare not to have any conflict of interest.

Introduction

Since their discovery, the opinion about oxysterols changed from cholesterol catabolites and wastes to key regulators of cell viability and proliferation, inflammatory processes, and cholesterol homeostasis. The brain and the peripheral cholesterol pools being separated by the blood-brain barrier (BBB), oxysterols, able to cross the BBB, are essential to control the *in situ* produced brain cholesterol pool and to maintain the brain and the neurovascular unit (NVU) cholesterol homeostasis. Moreover, oxysterols are the 'sensors' of both the peripheral and the central nervous system (CNS) cholesterol homeostasis and can, particularly for 24S-hydroxycholesterol (24S-OH-Chol) and 27-hydroxycholesterol (27-OH-Chol), testifying to some neurodegenerative disorders such as Alzheimer's disease (AD). In this review we describe the exchanges of oxysterols across the BBB, then shed the light on the mechanisms of cholesterol homeostasis controlled by oxysterols in the NVU. We also summarize their impacts on A β peptide burden in brain and transport across the BBB. Finally, we discuss a new potential therapeutic approach in AD, based on the use of 24S-OH-Chol.

1. The Blood-Brain Barrier, a compulsory pathway for oxysterols between the blood and the brain

The brain homeostasis is maintained and highly controlled by a natural barrier located in the brain microvessels that drastically restricts and controls the exchanges of molecules and cells between the brain compartment from the bloodstream. This barrier is named the blood-brain barrier (BBB). The BBB phenotype (Figure 1) held by the endothelial cells (ECs) is characterised by: (i) the presence of different junction complexes between the ECs such as tight junctions and adherens junctions which limit the paracellular transport of compounds (ii) a reduced aspecific transcytosis, the latter is mainly driven by specific transporters or receptors through clathrin and/or caveolae-dependent transcytosis; (iii) a restricted free diffusion of compounds due to metabolic enzymes such as cytochrome p450 enzymes (CYPs), monoamine oxidase (MAO), endothelin-converting enzymes (ECEs), and efflux pumps (P-glycoprotein, Multidrug Resistance Proteins (MRPs), etc) (for review, (Sweeney et al. 2019). The appearance and the maintenance of the BBB phenotype are due to the cell-cell communication between ECs and their neighbour cells in brain microvessels (brain pericytes,

astrocytes and neurons (Daneman et al. 2010)). These four cell types form a physiological and functional cell assembly referred to as the NeuroVascular Unit (NVU)(Muio et al. 2014).

Through these control systems, the BBB also keeps the brain isolated from the bloodstream in terms of cholesterol metabolism, despite the important needs of cholesterol to ensure the neuronal functions (Saher et al. 2005). Indeed, brain represents 2% of the body weight but contains nearly 25% of the overall content of cholesterol, most of the cholesterol is enriched in axonal myelin sheets. Free cholesterol exchanges across the BBB are very limited (Chobanian and Hollander 1962, Chobanian et al. 1962, Spady et al. 1987, Wilson 1970), and are mediated by the Low-Density Lipoproteins (LDLs) transcytosis from blood to brain across the BBB (Candela et al. 2008, Dehouck et al. 1997). This low cholesterol intake from the bloodstream is compensated by a significant *de novo* synthesis of cholesterol in astrocytes that transfer this lipid to neurons by producing High-Density Lipoprotein (HDL)-like particles (a process detailed in the part 2.). CNS elimination of cholesterol is subsequently possible after its oxidation into 24S-OH-Chol by subsets of neurons expressing the enzyme CYP46A1. This oxysterol is mainly eliminated (6 mg/day) in the bloodstream through the BBB to be metabolised by the liver into bile acids (**Figure 2.A**,(Bjorkhem 2006, Bjorkhem et al. 2019)). Thus, contrary to cholesterol and according to the hypothesis developed by Meaney and colleagues, oxysterols are able to cross the BBB thanks to the presence of a hydroxyl group on the carboxy-terminal tail of cholesterol molecule which provides a better diffusion through the plasma membrane by reducing the hydrophobic interaction with the phospholipids (**Figure 2.B**,(Meaney et al. 2002)). Despite that 24S-OH-Chol is mainly produced in brain, CYP46A1 is also expressed in brain capillary ECs, suggesting a small but existing local production of 24S-OH-chol at the BBB level (Schweinzer et al. 2011).

27-hydroxycholesterol (27-OH-Chol), the major oxysterol in plasma and produced in the liver by the enzyme sterol-27 hydroxylase (or CYP27A1), is known to enter the brain (5 mg/day) to be metabolised by neurons (Cali and Russell 1991, Heverin et al. 2005, Lutjohann et al. 1996, Meaney et al. 2004). After modifications by the enzymes CYP7A1/B1, CYP47A1 and HSB3B7, 27-OH-Chol is transformed into 7 α -hydroxy-3-oxo-4-cholestenoic acid (7 α -OH-4-CA), this metabolite then exits the brain (2 mg/day) to be used in liver for the production of primary bile acid (**Figure 2.B**,(Heverin et al. 2004, Meaney et al. 2007)). Therefore, the BBB is daily crossed by 24S-OH-Chol and 27-OH-Chol which are considered as 'sensors' of the cholesterol

homeostasis, and this barrier is responsible of the existence of physiological ratios of 24S-OH-Chol/ 27-OH-Chol: 0.5 (1/2) in blood and in 10 (10/1) in brain (Leoni et al. 2003). In contrast, for some minor circulating oxysterols such as 25-hydroxycholesterol (25-OH-Chol), their concentration is too low to measure any transport across the BBB (lower than 10 ng/mL, between 30 and 150 ng/mL for the major oxysterols such as 24S-OH-Chol and 27-OH-Chol,(Griffiths et al. 2006)). However, the wide expression of the cholesterol 25-hydroxylase (CH-25-H), which converts cholesterol into 25-OH-Chol, could suggest a global production of 25-OH-Chol and a possible transport across the BBB following concentration gradient which remains undetermined yet (Bjorkhem and Diczfalusy 2002, Breuer and Bjorkhem 1995, Lund et al. 1999).

Thus, the BBB is crossed by constant passive fluxes of oxysterols due to their permissive chemical structures and following their concentration gradient. It is also described that oxysterols can be shuttled by HDLs across the BBB as demonstrated for 24S-OH-Chol (Panzenboeck et al. 2002, Saint-Pol et al. 2012). Another study using rats and oatp2-expressing oocytes highlighted the possible role of organic anion transporter transporting polypeptide 2 (oatp2) in the transport of 24S-OH-Chol out of the brain (Ohtsuki and Terasaki 2007).

2. Oxysterols and the regulation of the cholesterol homeostasis in the NVU

As natural endogenous agonists of the Liver X nuclear Receptors (LXRs), oxysterols stimulate the expression of the LXR target genes involved in the regulation of the cholesterol homeostasis. The most characterized LXR target genes are *Abca1*, *Abcg1*, *Apoa-1* and *ApoE* coding for the proteins ATP Binding Cassette sub-family A member 1 (ABCA1), ABCG1 and the apolipoproteins A-1 (ApoA-1) and ApoE, respectively (Hu et al. 2010). These proteins mediate the reverse cholesterol transfer (RCT) from cells to (apo)lipoproteins and are key regulators of cellular cholesterol homeostasis (Figure 3). The role of oxysterols in cholesterol homeostasis in the NVU has been studied first focusing on the brain part (astrocytes and neurons), and more recently at the BBB levels, since brain microvessels and brain pericytes express LXR nuclear receptors and their target genes (Akanuma et al. 2008, Gosselet et al. 2009, Panzenboeck et al. 2002, Saint-Pol et al. 2013, Saint-Pol et al. 2012).

In brain, 24S-OH-Chol is involved in a regulatory loop between astrocytes and neurons to control brain cholesterol homeostasis. In fact, the excess of cholesterol in neurons is

metabolized into 24S-OH-Chol which regulates the cholesterol production *de novo* (through a negative control on the 3-Hydroxy-3-MethylGlutaryl-Coenzyme A (HMG-CoA) reductase) and increases the expression of ABCA1, ABCG1 and ApoE in a dose-dependent manner as demonstrated in primary astrocytes and CCF-STTGA astrocytoma cell line (Abildayeva et al. 2006). ABCA1 initiates the RCT in astrocytes by transferring cholesterol to non-lipidated ApoE to form discoid ApoE. This lipidation statement provides a second step of cholesterol transfer *via* ABCG1 leading to spheroid ApoE called 'HDL-like' particles because their density is close to circulating HDL density. The cholesterol and cholesterol esters in HDL-like particles are then uptaken by neurons *via* the Low-Density Lipoprotein Receptor (LDLR) or Low-Density Lipoprotein Receptor-related Proteins (LRPs) to ensure the synaptic communications, the electric isolation and repair of myelin sheets (Hirsch-Reinshagen and Wellington 2007, Pfrieger 2003, Saher et al. 2005). According to former studies performed in rats by $^{18}\text{O}_2$ inhalation, about 2/3 of brain cholesterol produced *de novo* is converted into 24S-OH-Chol per hour, highlighting the importance of the 24S-OH-Chol regulatory loop in brain cholesterol homeostasis (Bjorkhem and Lewenhaupt 1979, Bjorkhem et al. 1997). 27-OH-Chol has been recently described to modulate the cholesterol homeostasis in astrocytes in a study performed with C6 glioma cells. This oxysterol decreased free cholesterol and cholesterol ester content and the expression of HMG-CoA reductase, SREBP1-a and LDLR in a dose-dependent manner (An et al. 2017).

Concerning the BBB, we and others characterized *in vitro* the impact of both 24S-OH-Chol and 27-OH-Chol in cholesterol homeostasis in brain microvessel ECs and in brain pericytes. Both oxysterols increase the expression of ABCA1 and ABCG1 in a dose-dependent manner in porcine (Panzenboeck et al. 2002, Panzenboeck et al. 2006) and bovine primary brain capillary ECs (Saint-Pol et al. 2013), as well as in human brain-like endothelial cells (Saint-Pol and Gosselet, unpublished data). Since the presence of tight junctions in the apical part of brain microvessel ECs, Panzenboeck's team studied the role of 24S-OH-Chol and 27-OH-Chol in ABCA1 and ABCG1 polarization. In a non-stimulated condition, ABCA1 is mainly localized at the abluminal side of the ECs (Panzenboeck et al. 2006, Schweinzer et al. 2011), whereas ABCG1 is located in both sides (Kober et al. 2017). Oxysterols stimulation of ABCG1 expression does not impact its location (Kober et al. 2017), but the increased expression of ABCA1 is associated with its relocation in both luminal and abluminal sides of the ECs (Panzenboeck et al. 2002). In brain pericytes, 24S-OH-Chol (Saint-Pol et al. 2012) and 27-OH-

Chol (Saint-Pol et al., unpublished data) increase the expression of only ABCA1. ABCG1 seems to be neither expressed nor induced by the treatment in these cells. In terms of RCT, the increased expression of ABCA1 is correlated with an increase of cholesterol efflux from brain pericytes to ApoA-1 (secreted from ECs), ApoE3, ApoE4 and HDL. The absence of ABCG1 in brain pericytes can be compensated by the presence of Scavenger Receptor B member 1 (SR-B1) and ABCG4, both involved in the release of free cholesterol and cholesterol ester to discoid ApoE or HDL (Do et al. 2012, Rigotti et al. 2003) but their expression is modified neither by 24S-OH-Chol nor by 27-OH-Chol ((Saint-Pol et al. 2012) and unpublished data). In ECs, 24S-OH-Chol and 27-OH-Chol increase the total cholesterol efflux to ApoA-1 and HDL₃ through the increase of ABCA1 and ABCG1 expression and function (Kober et al. 2017, Panzenboeck et al. 2006, Saint-Pol et al. 2013). As the ECs are in direct contact with the bloodstream, the RCT is slightly different from the one previously described in the brain (**Figure 3**). ABCA1 initiates the RCT through the transfer of free cholesterol and cholesterol ester to ApoA-1 to form pre- β HDL particles, the latter are then loaded with cholesterol ester to form immature HDL₃. ABCG1 finishes the RCT by releasing cholesterol to HDL₃ *via* the ApoM exposed at their surface (Kober et al. 2017). The neoformed HDL₂ particles is then uptaken by cells to access the pool of cholesterol they carry, or are metabolized in the liver. Hence, oxysterols control cholesterol homeostasis within the NVU through the activation of LXR pathway and the function of their target genes in RCT, and according to the surface represented by the BBB (18 m² representing about 600 km of brain microvessels inside the brain), the maintenance of the NVU cholesterol homeostasis contributes (at least in part) to the regulation and the maintenance of brain cholesterol homeostasis.

3. The NVU, oxysterols and Alzheimer's disease

AD is a neurodegenerative disease characterised by two major hallmarks: (i) hyperphosphorylation and/or abnormal phosphorylation of tau proteins leading to the appearance of neurofibrillary tangles (NFTs); (ii) an altered clearance of amyloid- β (A β) peptides which accumulate around brain microvessels of the BBB and in the brain parenchyma, thus leading to the formation of amyloid plaques. NFTs and A β plaques promote neuronal cell death and the progressive cognitive decline (Querfurth and LaFerla 2010). Despite some recent data suggesting that the AD-associated tauopathy is responsible

for vascular damages and for the BBB breakdown (Bennett et al. 2018, Blair et al. 2015), the vascular side of AD is commonly associated with a defective clearance of A β peptides through the BBB and their accumulation in perivascular spaces leading to a cerebral amyloid angiopathy (CAA)(Mawuenyega et al. 2010, Thal et al. 2008). Furthermore, the BBB is involved in bidirectional A β peptide exchanges between the brain and the blood ((Gosset et al. 2013); **Figure 4, bright side**). Influx of A β peptides across the BBB, i.e. their entry in the brain, is driven by the receptor for advanced glycation end-products (RAGE) which is expressed in the luminal side of the brain microvessel ECs, and is restricted by several efflux pumps including P-glycoprotein (ABCB1) and breast cancer resistance protein (BCRP or ABCG2,(Candela et al. 2010)). Efflux of A β peptides across the BBB is dependent of ABCG4 and a possible tandem LRP1-ABCB1 (Candela et al. 2015, Do et al. 2012, Storck et al. 2018). LRP1 is 1000 times more expressed in brain pericytes than in ECs (Gosset et al. 2009, Candela et al. 2015) and is involved in A β peptide accumulation in brain pericytes (Candela et al. 2015, Wilhelmus et al. 2007). LRP1 mediates also A β -ApoE complexes from the brain (Bachmeier et al. 2013). Moreover, Panzenboeck's team demonstrated that porcine primary brain capillary ECs expressed the precursor of A β peptide (APP) and the secretases responsible of amyloidogenic and non-amyloidogenic cleavage of APP, ECs have a slight but local production of A β peptides (Schweinzer et al. 2011).

Plethora of studies have highlighted the close relationship between AD and brain cholesterol metabolism (Vance 2012). ABCA1, the major LXR target gene, was thought to be involved in A β uptake and /or degradation since *Lxrs* or *Abca1*-deficient AD mice showed an increased A β peptide deposition in brain parenchyma (Koldamova R. et al. 2005a, Wahrle et al. 2005, Zelcer et al. 2007). On the contrary, *Abca1* knock-in AD mice or AD mice treated with LXR agonists demonstrated a decreased A β burden in brain associated with a progressive cognitive recovery (Burns et al. 2006, Donkin et al. 2010, Fitz et al. 2010, Koldamova R. P. et al. 2005b, Riddell et al. 2007, Wahrle et al. 2008). We investigated the effect of LXR stimulation by both 24S-OH-Chol and 27-OH-Chol in soluble A β peptide transport across the brain microvessel ECs and A β uptake by brain pericytes and clearly demonstrated that ABCA1 is not involved in these processes despite an increased expression (Saint-Pol et al. 2013, Saint-Pol et al. 2012). Interestingly, 24S-OH-Chol and 27-OH-Chol decreased A β peptide influx across brain microvessel ECs through an increase of ABCB1 protein expression and function. As ABCB1 is not linked to the LXR pathway, we concluded

that oxysterols may regulate ABCB1 expression through an indirect pathway that remains to be investigated (Saint-Pol et al. 2013).

In terms of APP cleavage in brain capillary ECs, 24S-OH-Chol and 27-OH-Chol decreased BACE1 expression, the secretase involved in the initiation of the amyloidogenic cleavage of APP (and the further production of A β peptides) and promoted the release of sAPP α in the basolateral side (i.e. brain side), a soluble fragment associated with the non-amyloidogenic cleavage of APP (Schweinzer et al. 2011). However, these findings are in opposition with previous data obtained in SH-SY5Y neuroblastoma cells where 24S-OH-Chol and 27-OH-Chol differentially regulated APP processing, 27-OH-Chol regulation was in favour of A β ₁₋₄₂ production (Prasanthi et al. 2009) and 24S-OH-Chol altered intracellular APP trafficking which is retained in the endoplasmic reticulum (Urano et al. 2013), thus decreasing A β production. Since 24S-OH-Chol/27-OH-Chol ratios in brain and in blood are altered in AD in the benefit of 27-OH-Chol (Bjorkhem 2006), the suggested amyloidogenic regulation of 27-OH-Chol on APP processing and the increased expression of CYP27A1 in AD patient brains (Testa et al. 2016) could be in favour of an increased production of A β peptides. Further investigations would be therefore needed to clarify this APP processing regulation by oxysterols. (Figure 4, dark side)

The rate of circulating 24S-OH-Chol decreases with age and is altered in AD patients as reported by an altered 24S-OH-Chol/27-OH-Chol ratios observed in AD patients compared with healthy people (Bjorkhem 2006). This alteration depends on the stage of the disease. Indeed, it has been previously demonstrated that the plasma 24S-OH-Chol concentration is increased in early stages of AD (Lutjohann et al. 2000), whereas this concentration decreased following the progression of the disease (Bretillon et al. 2000a, Bretillon et al. 2000b). These variations are correlated with the observed alteration of neuronal CYP46A1 activity and expression in AD patient brains (Bogdanovic et al. 2001, Testa et al. 2016). Moreover, the inhibition of Cyp46a1 in APP23 mouse hippocampus *in vivo* by a small hairpin RNA (shRNA) led to an increase of neuronal cholesterol levels, more apoptotic cell death and the production of A β peptides and phosphoTau (Djelti et al. 2015). A recent study shed the light on the effect of efavirenz, an anti-retroviral drug used as anti-HIV, on the CYP46A1 activity in 5xFAD mice. The treated mice showed improved cognitive functions in association with a decreased A β burden (Mast et al. 2017). However, once the treatment stopped, the activation of CYP46A1 and the brain cholesterol turnover stopped, promoting the long-term

spatial memory defects and short-term memory troubles (Mast et al. 2017). Moreover, even these potential benefit effects in the CNS, efavirenz treatments in mice have been reported to promote a progressive BBB disruption (increased BBB permeability and decreased expression of claudin-5) associated with vascular side effect such as stroke (Bertrand et al. 2016). The use of efavirenz or the development of chemical analogues with lower side effects, and particularly on the BBB, needs obviously to be optimized. However, CYP46A1 activity in AD appears to be crucial not only to maintain brain and the NVU cholesterol homeostasis, but also to prevent cognitive decline and the apparition of AD hallmarks in brain and altered A β peptide clearance, identifying therefore CYP46A1 as a new promising therapeutic target in AD.

Conclusion

The BBB, which is included in a bigger cellular complex referred to as the NVU (including brain pericytes, astrocytes and neurons of the perivascular spaces), is the main road of oxysterols exchanges, and oxysterols ratios determined by this barrier are key indicators to distinguish physiological and neurodegenerative contexts. Oxysterols, and particularly 24S-OH-Chol and 27-OH-Chol, contribute to control and maintain the complex brain cholesterol homeostasis which is in part correlated with the maintenance of the NVU cholesterol homeostasis. The maintenance of stable concentrations of 24S-OH-Chol and physiological 24S-OH-Chol/27-OH-Chol ratios by targeting CYP46A1 seems to improve not only the cognitive functions and to lessen A β burden in brain, but also to keep the physiological regulation of A β peptide exchanges through the BBB, allowing a potential double-sided therapeutic approach in AD which could limit the perivascular defects of the disease.

FIGURES

Figure 1: Induction and maintenance of the blood-brain barrier (BBB) main features on brain microvessel endothelial cells. AJs: Adherens Junctions, JAMs: Junctional Adhesion Molecules, LAMs: Leukocyte Adhesion Molecules, LDLR: Low-Density Lipoprotein Receptor, NVU: NeuroVascular Unit, PDGF: Platelet-Derived Growth Factor, TJs: Tight Junctions.

Figure 2: Exchanges of oxysterols across the BBB. Major passive fluxes of oxysterols (A) and the actual hypothesis of oxysterol passive diffusion across the membrane leaflets (B). 7 α -OH-4-CA: 7 α -hydroxy-3-oxo-4-cholestenoic acid, 24S-OH-Chol: 24S-hydroxycholesterol, 27-OH-Chol: 27-hydroxycholesterol, AJs: Adherens Junctions, BM: Basement membrane, CYP7A1/B1: cholesterol 7 α -hydroxylase, CYP27A1: sterol 27-hydroxylase, CYP46A1: cholesterol 24-hydroxylase, EC: Endothelial Cell, HSB3B7: 3 β -hydroxy-C27-steroid deshydrogenase/hydroxylase, P: Pericyte, TJs: Tight Junctions.

Figure 3: Regulation of cholesterol homeostasis in the NVU by oxysterols and ratio 24S-OH-Chol/27-OH-Chol (R) in plasma and brain. 24S-OH-Chol: 24S-hydroxycholesterol, 27-OH-Chol: 27-hydroxycholesterol, ABCs: ATP-Binding Cassettes, AJs: Adherens Junctions, Apo: Apolipoprotein, CETP: Cholesteryl Ester Transfer Protein, CYP46A1: cholesterol 24-hydroxylase, EC: Endothelial Cell, HDL: High-Density Lipoprotein, HMG-CoA: 3-hydroxy-3-methylglutaryl Coenzyme A, HMG-CoAR: 3-hydroxy-3-methylglutaryl Coenzyme A Reductase, LDLR: Low-Density Lipoprotein Receptor, LRP: Low-density lipoprotein receptor-Related Peptides, P: Pericyte, SR-B1: Scavenger Receptor class B member 1, TJs: Tight Junctions.

Figure 4: 24S-OH-Chol, 27-OH-Chol and Alzheimer's disease - bright and dark sides within the NVU. 24S-OH-Chol: 24S-hydroxycholesterol, 27-OH-Chol: 27-hydroxycholesterol, A β : β -amyloid, ABCs: ATP-Binding Cassettes, ABCB1: ATP-Binding Cassette sub-family B member 1 (i.e. P-glycoprotein or P-gp), ABCC1: ATP-Binding Cassette sub-family C member 1 (i.e. Multidrug Resistance-associated Protein 1 or MRP1), ABCG2: ATP-Binding Cassette sub-family G member 2 (i.e. Breast Cancer Resistance Protein or BCRP), AJs: Adherens Junctions, APP: Amyloid Precursor Peptide, BACE1: β -site APP Cleaving Enzyme 1, BBB: Blood-Brain

Barrier, APP- β CTF: APP- β CarboxyTerminal Fragment, CYP46A1: cholesterol 24-hydroxylase, EC: Endothelial Cell, LRP1: Low-density lipoprotein receptor-Related Peptide 1, P: Pericyte, RAGE: Receptor for Advanced Glycation End-products, TJs: Tight Junctions.

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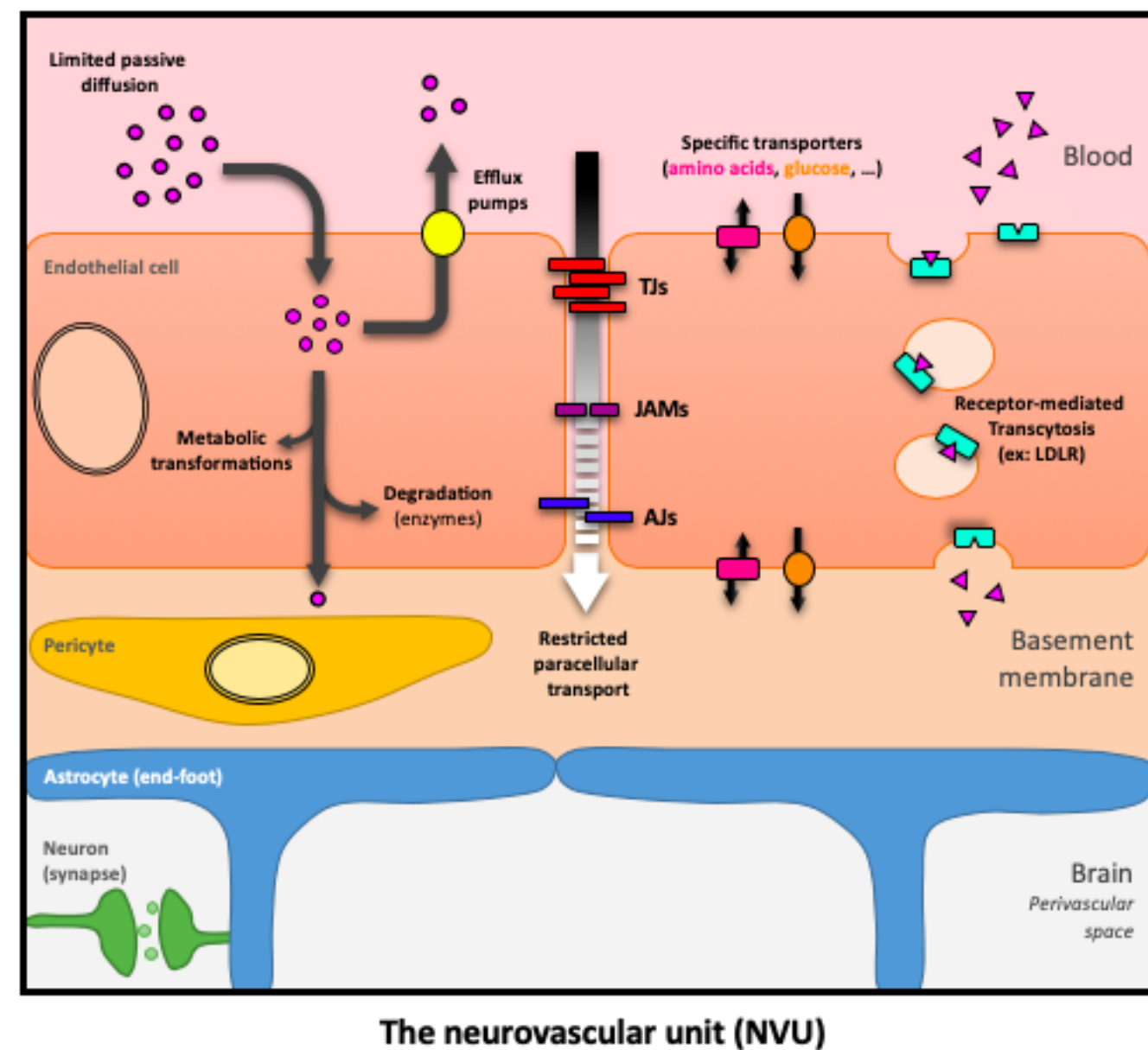
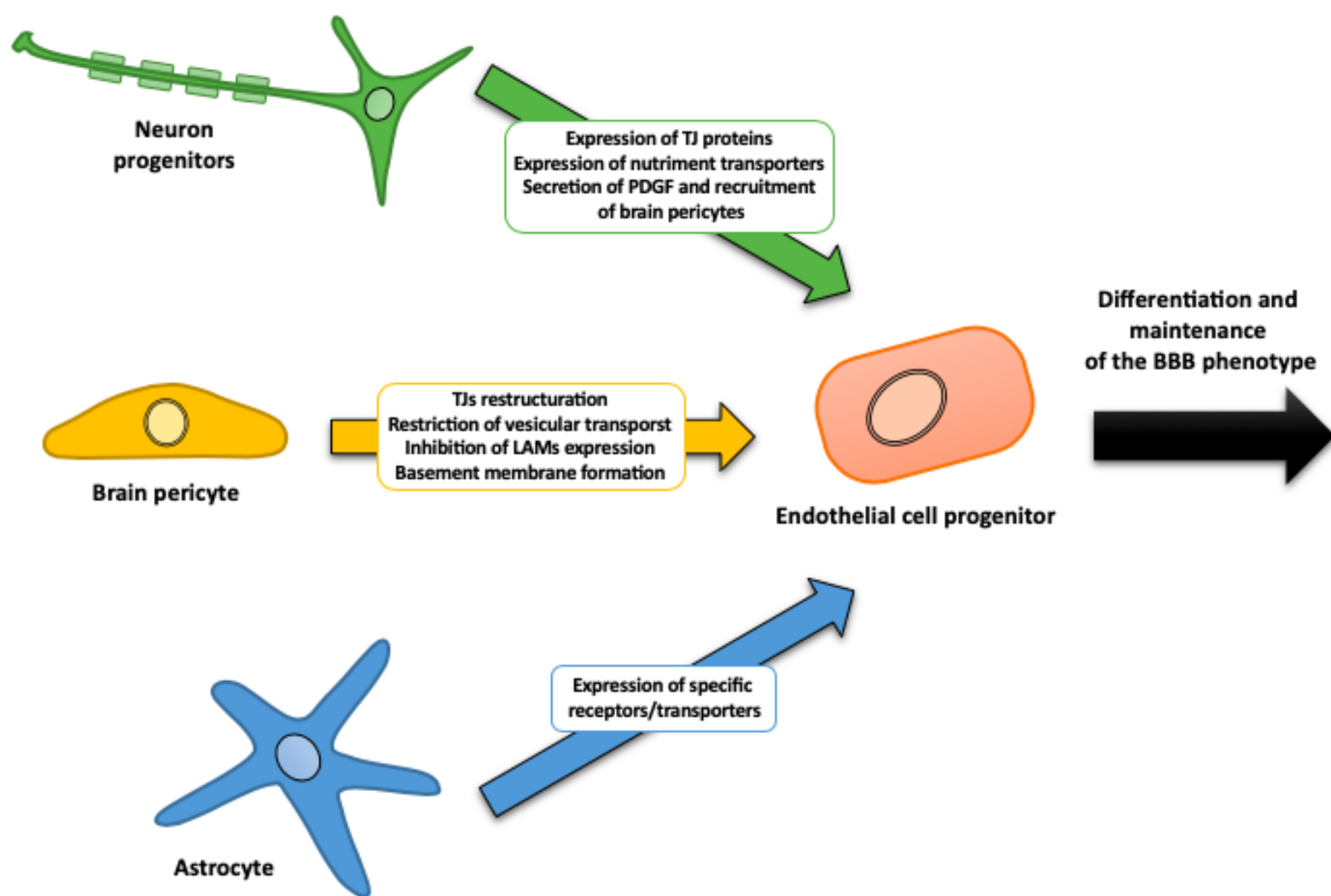
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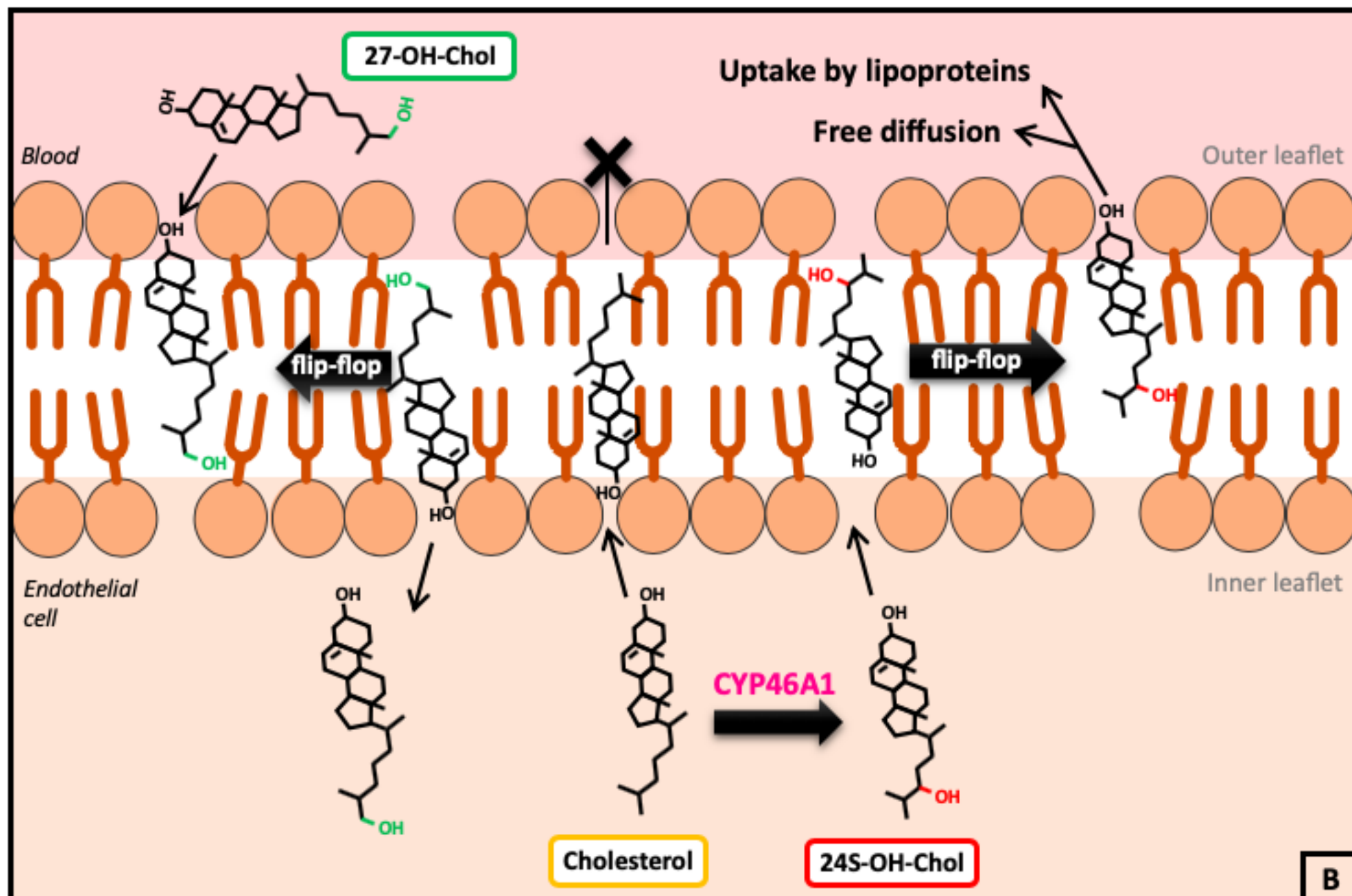
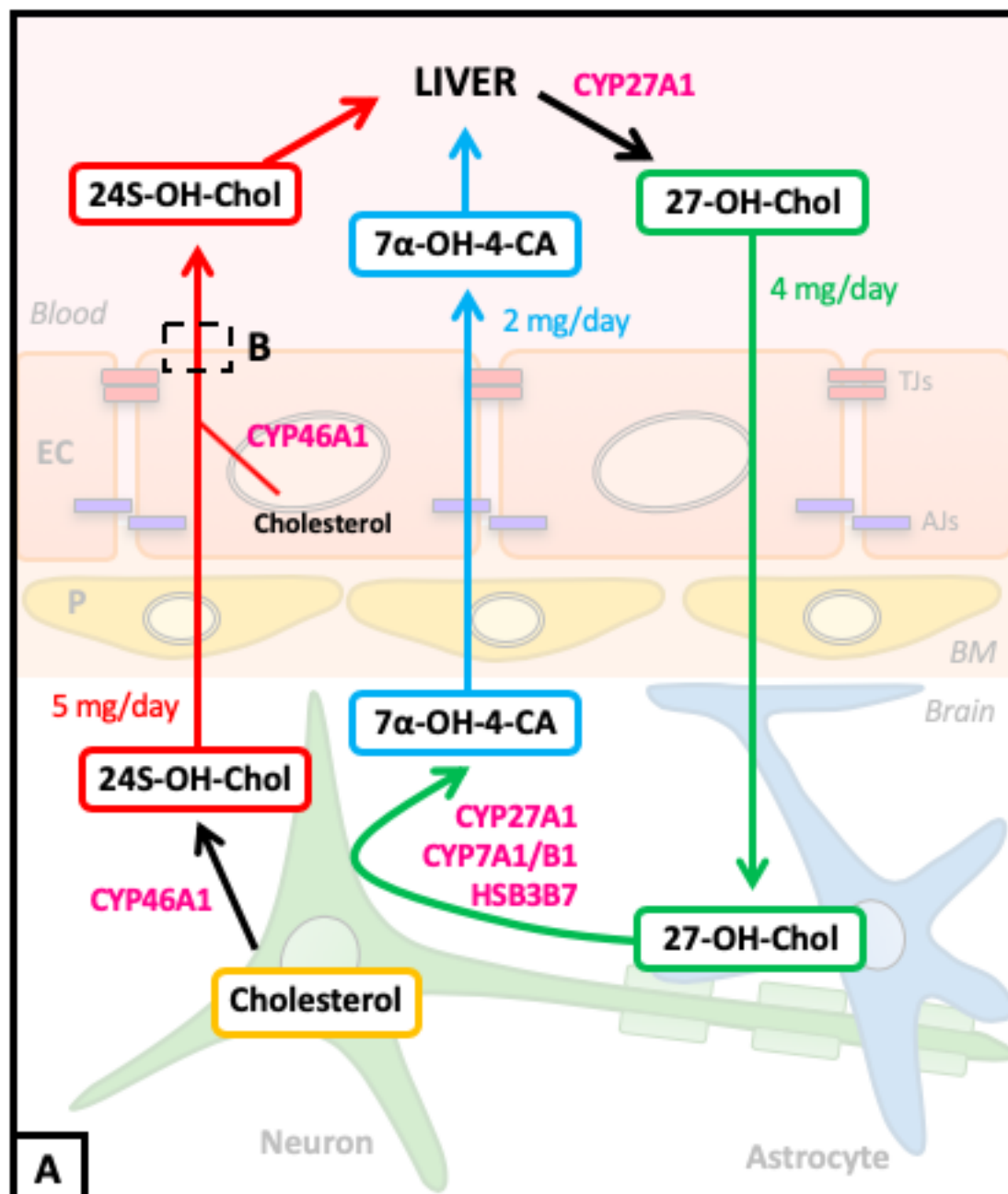
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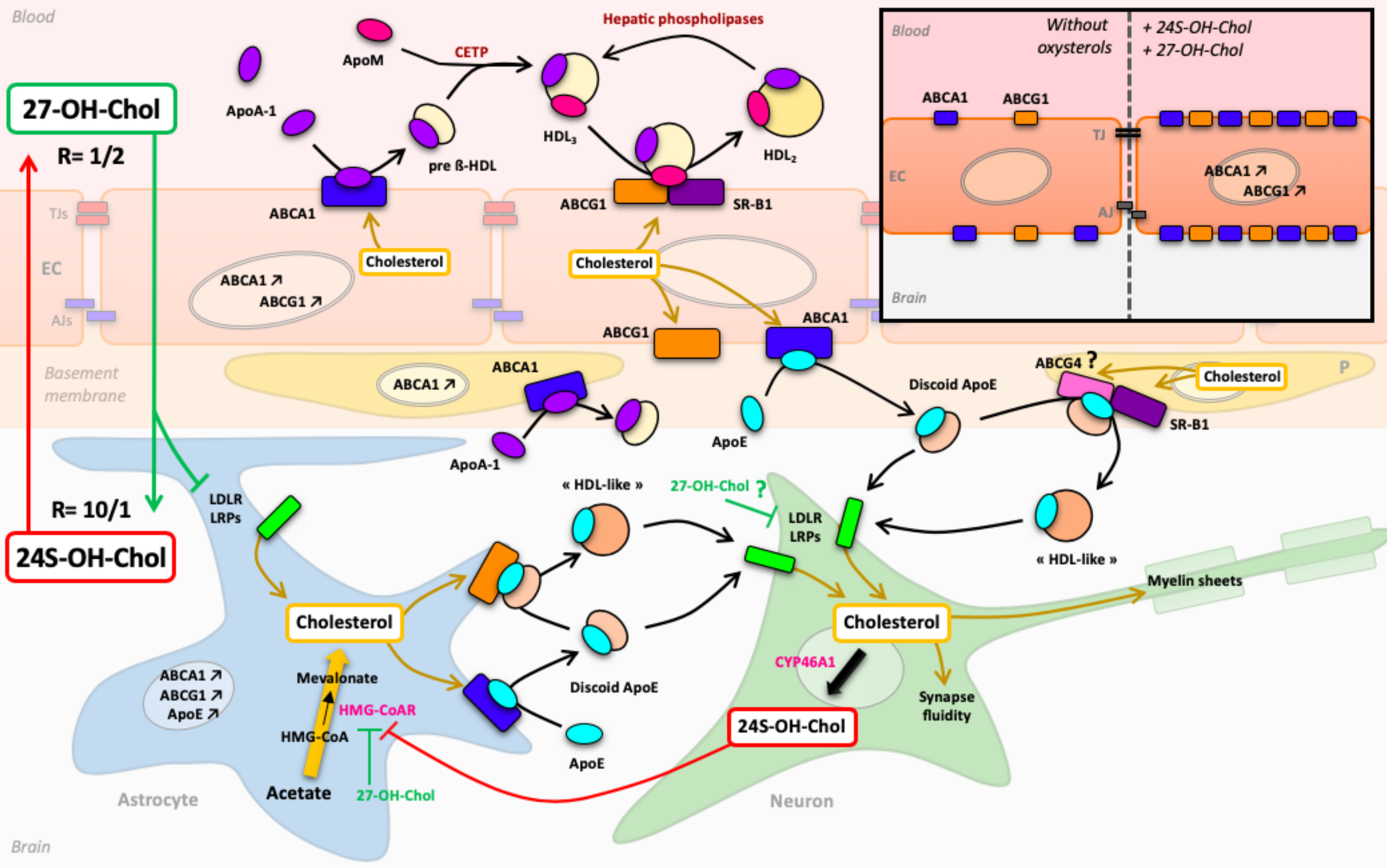
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Blood

Healthy condition

Alzheimer's disease

