Neuro-Oxysterols and Neuro-Sterols as Ligands to Nuclear Receptors, G Protein-Coupled Receptors, Ligand-Gated Ion Channels and other Protein Receptors

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Abstract

The brain is the most cholesterol rich organ in the body containing about 25% of the body's free cholesterol. Cholesterol cannot pass the blood brain barrier and be imported or exported directly, instead it is synthesised in situ and metabolised to oxysterols, oxidised forms of cholesterol, which can pass the blood brain barrier. 24S-Hydroxycholesterol is the dominant oxysterol in brain after parturition but during development a myriad of other oxysterols are produced which persist as minor oxysterols after birth. During both development and in later life, oxysterols and other sterols interact with a variety of different receptors, including nuclear receptors e.g. liver X receptors; membrane bound G protein-coupled receptors e.g. smoothened; the endoplasmic reticulum resident proteins e.g. INSIG (insulin induced gene), or the cholesterol sensing protein SCAP (SREBP cleavage activating protein); and the ligand-gated ion channel N-methyl-D-aspartate receptors found in nerve cells. In this review we summaries the different oxysterols (neuro-oxysterol) and sterols (neuro-sterols) found in the central nervous system whose biological activity is transmitted via these different classes of protein receptors.

Introduction

It has been known for decades that C_{18}-C_{21} steroids are ligands to nuclear receptors and since the 1990's that oxysterols are ligands to "orphan", now "adopted", nuclear receptors (Evans & Mangelsdorf, 2014). Importantly, nuclear receptors are expressed in brain where oxysterols are also abundant (Warner & Gustafsson, 2015). These receptors work via regulating gene and hence protein expression.*N* -methyl-D-aspartate receptors (NMDARs) are expressed in nerve cells and work on a much shorter time scale. They are ligand-gated ion channels activated by the neurotransmitter glutamate, critical to the regulation of excitatory synaptic function. NMDARs are modulated by excitatory neurosteroids and by the neuro-oxysterol 24S-hydroxycholesterol (24S-HC, Figure 1, see Supporting Information Table S1 for systematic and common names and their abbreviations) (Paul et al., 2013).

For simplicity, in this article we use the term oxysterol to cover oxidised forms of cholesterol and its precursors (Javitt, 2008; Schroepfer, 2000), and use the term sterol to specifically embrace cholesterol and its cyclic precursors. By extension neuro-oxysterols and neuro-sterols are the respective terms to define oxysterols and sterols found in the central nervous system (CNS). The sterol definition is at variance with the formal definition of sterols by Lipid Maps to include*all* molecules based on the cyclopentanoperhydrophenanthrene skeleton and ring-opened versions thereof (Fahy et al., 2005).

G protein-coupled receptors (GPCR) are cell membrane receptors that are activated by molecules outside the cell and activate signal transduction pathways within the cell. Cell membranes are rich in cholesterol and membrane cholesterol plays an important role in GPCR structure and function (Sengupta & Chattopadhyay, 2015). Several cholesterol interaction sites have been identified in GPCRs whose occupancy may modulate

GPCR activity. Smoothened (SMO), a member of the Frizzled-class of GPCRs and a critical component of the hedgehog (Hh) signalling pathway, has a cholesterol binding site within its extracellular cysteine rich domain (CRD), mutational modification of which impairs the ability of SMO to transmit Hh signals (Byrne et al., 2016). The Hh pathway is critical for tissue patterning during development and abnormal function is associated with birth defects and cancer (McCabe & Leahy, 2015). Defective Hh signalling is implicated in Smith-Lemli-Opitz syndrome (SLOS) which presents, not only with dysmorphology, but also learning and behavioural problems, highlighting the importance of Hh signalling in brain (Cooper et al., 2003; Kelley et al., 1996). Numerous oxysterols have also been found to activate the Hh signalling pathway by binding to the CRD of SMO (Nedelcu, Liu, Xu, Jao & Salic, 2013; Qi, Liu, Thompson, McDonald, Zhang & Li, 2019; Raleigh et al., 2018), many of these are found in brain and are hence neuro-oxysterols (Yutuc et al., 2020). GPCR183, also known as Epstein Barr Virus Induced GPCR or EBI2, is involved in the trafficking of immune cells towards their EBI2 ligands i.e. oxysterols with a dihydroxycholesterol (diHC) structure, where one additional hydroxy group is at C-7α and the second on the side-chain (Hannedouche et al., 2011; Liu et al., 2011). As T-cell trafficking plays a major role in the neurodegenerative autoimmune disease multiple sclerosis and in its animal model, experimental autoimmune encephalitis (EAE), GPCR183 and neuro-oxysterols have been studied in this regard (Duc, Vigne & Pot, 2019). 7α,25-Dihydroxycholesterol (7α,25-diHC) and 7α,(25R)26-dihydroxycholesterol (7α,(25R)26-diHC, also known as 7α,27-dihdroxycholesterol, note, if stereochemistry at C25 is not indicated it is assumed to be 25R) are both GPCR183 agonists present in brain (Griffiths et al., 2019a) and 7α,25-diHC has been found to be increased in spinal cord during EAE development (Wanke et al., 2017).

All cells in vertebrates express the enzymatic machinery to synthesise cholesterol. The cholesterol biosynthesis pathway, also known as the mevalonate pathway, (see http://www.lipidmaps.org/pathways/pathway_lipids_list.php for exact details) is regulated by the master transcription factor SREBP-2 (sterol regulatory element-binding protein 2) which also regulates the expression of the low density lipoprotein receptor (LDLR) (Horton, Goldstein & Brown, 2002). When cholesterol levels are low, SREBP-2 is transported by SCAP (SREBP cleavage activating protein) from the endoplasmic reticulum to the Golgi where it is processed to its active form which translocates to the nucleus and activates target gene transcription, hence up-regulating cholesterol biosynthesis and import via LDLRs and restoring cholesterol levels in the cell (Goldstein, DeBose-Boyd & Brown, 2006). When cholesterol levels are elevated, cholesterol in the endoplasmic reticulum binds to SCAP which becomes tethered to the resident endoplasmic reticulum protein INSIG (insulin induced gene) and prevents transport of SREBP-2 to the Golgi for activation (Goldstein, DeBose-Boyd & Brown, 2006; Sun, Seemann, Goldstein & Brown, 2007). In this way cholesterol regulates its own biosynthesis and import via the LDLR. Oxysterols also inhibit cholesterol biosynthesis, but in this case, by binding to INSIG and tethering the SCAP - SREBP-2 complex in the Golgi (Radhakrishnan, Ikeda, Kwon, Brown & Goldstein, 2007). Although the turnover of cholesterol in the adult brain is slow (0.4% per day in mouse, 0.03% in human) the rate of synthesis is much higher during development (Dietschy & Turley, 2004). In the foetal mouse, the blood-brain barrier (BBB) is formed at E10 - E11 and after E11 - 12 the brain is the source of essentially all new sterols (Tint, Yu, Shang, Xu & Patel, 2006). Importantly, cholesterol 24-hydroxylase (cytochrome P450 46A1, CYP46A1) expression is low until E18, conserving cholesterol and restricting the biosynthesis of the neuro-oxysterol 24S-HC (Tint, Yu, Shang, Xu & Patel, 2006). CYP46A1 is the dominant cholesterol hydroxylase in brain and hydroxylation of cholesterol to 24S-HC is responsible for 50 - 60% of all cholesterol metabolism in adult brain (Russell, Halford, Ramirez, Shah & Kotti, 2009).

Other receptors for oxysterols include cytoplasmic oxysterol-binding protein (OSBP) and a family of proteins showing sequence homology to OSBP called OSBP-related (ORP) or OSBP-like (OSBPL) (Olkkonen & Hynynen, 2009). OSBP regulates lipid transport between the endoplasmic reticulum and Golgi and also can act as a sterol-dependent scaffold for protein phosphatases that dephosphorylate ERK (Olkkonen & Hynynen, 2009).

Nuclear Receptors

Liver X Receptors

Perhaps the most well studied activity of oxysterols is as ligands to nuclear receptors (Evans & Mangelsdorf, 2014; Warner & Gustafsson, 2015). Oxysterols are ligands to the liver X receptors α (LXRα, NR1H3) and β (LXRβ, NR1H2) (Janowski et al., 1999; Janowski, Willy, Devi, Falck & Mangelsdorf, 1996; Lehmann et al., 1997), the latter of which is highly expressed in brain (Wang, Schuster, Hultenby, Zhang, Andersson & Gustafsson, 2002). The most potent oxysterols are ones in which the side-chain of cholesterol has been modified by hydroxylation to give monohydroxycholesterols (HC), epoxidation to give 24S,25-epoxycholesterol (24S,25-EC) (Janowski et al., 1999; Janowski, Willy, Devi, Falck & Mangelsdorf, 1996; Lehmann et al., 1997) or carboxylation to give a carboxylic acid (Ogundare et al., 2010; Song & Liao, 2000; Theofilopoulos et al., 2014), although other oxysterols also activate these receptors (Segala et al., 2017).

Following birth, the dominant neuro-oxysterol in brain is 24S-HC (Bjorkhem, 2007; Dietschy & Turley, 2004; Ercoli & Ruggieri, 1953). This is a ligand to both the LXRs. Surprisingly, the *cholesterol 24-hydroxylase* knock-out (*Cyp46a1*$^{-/-}$) mouse has a mild phenotype but does show defective motor learning and *in vitro* studies indicate impaired hippocampal long-term potentiation (LTP) (Kotti, Ramirez, Pfeiffer, Huber & Russell, 2006; Lund, Xie, Kotti, Turley, Dietschy & Russell, 2003). Thanks to the tight regulation of cellular cholesterol levels by cholesterol itself (Goldstein, DeBose-Boyd & Brown, 2006), the cholesterol levels in brain of the *Cyp46a1*$^{-/-}$ mouse are not distorted (Lund, Xie, Kotti, Turley, Dietschy & Russell, 2003; Meljon, Wang & Griffiths, 2014). However, Kotti et al found that a reduced rate of production of cholesterol precursors, specifically geranylgeraniol, as a consequence of an absence of CYP46A1, was the explanation for impaired hippocampal LTP (Kotti, Ramirez, Pfeiffer, Huber & Russell, 2006).

Before birth, 24S,25-EC is the major neuro-oxysterol (Theofilopoulos et al., 2013; Wang et al., 2009), and can be formed by at least two pathways. In the cholesterol biosynthesis pathway, squalene epoxidase (SQLE) introduces a 2,3-epoxy group into squalene, the resultant 3S-squalene-2,3-epoxide is then cyclised to lanosterol by the enzyme lanosterol synthase (LSS, Figure 2). If levels of 3S-squalene-2,3-epoxide are elevated squalene epoxidase can introduce a second epoxy group to give 3S-squalene-2,3;22,23-dioxide which is metabolised in parallel to 3S-squalene-2,3-epoxide by the same enzymes (except 24-dehydrocholesterol reductase, DHCR24) to give 24S,25-EC rather than cholesterol (Gill, Chow & Brown, 2008; Nelson, Steckbeck & Spencer, 1981). The second pathway involves oxidation of desmosterol to give 24S,25-EC. In brain the likely catalyst is CYP46A1 expressed in neurons (Goyal, Xiao, Porter, Xu & Guengerich, 2014), however, the reaction can also proceed in fibroblasts raising the possibility of the involvement of an alternative catalyst, or CYP46A1 expression in these cells (Saucier, Kandutsch, Gayen, Nelson & Spencer, 1990).

Theofilopoulos and colleagues have shown the importance of 24S,25-EC in the neurogenesis of midbrain dopaminergic neurons through activation of the LXRs (Theofilopoulos et al., 2013). They showed that midbrain progenitor cells derived from *Lxr* double knockout (*Lxr* $\alpha^{-/-}$*Lxr* $\beta^{-/-}$) mouse embryos have reduced neurogenic capacity (Sacchetti et al., 2009), while exogenous 24S,25-EC promoted dopaminergic neurogenesis in midbrain progenitor cells derived from wild type embryos (Theofilopoulos et al., 2013). 24S,25-EC also promoted dopaminergic differentiation of embryonic stem cells, suggesting that 24S,25-EC, or perhaps more chemically stable LXR ligands, may contribute to the development of cell replacement and regenerative therapies for Parkinson's disease, a disease characterised by the loss of dopaminergic neurons (Theofilopoulos et al., 2013). In the *CYP46A1* transgenic mouse (*CYP46A1* tg) overexpressing human CYP46A1, the concentration of 24S,25-EC is elevated in the developing ventral midbrain (Theofilopoulos et al., 2019). Theofilopoulos et al exploited this mouse to show an increase in midbrain dopaminergic neurons *in vitro* and *in vivo*.Importantly, 24S-HC which is also elevated in *CYP46A1* tg mouse developing midbrain does not affect *in vitro* neurogenesis of midbrain dopaminergic neurons (Theofilopoulos et al., 2019; Theofilopoulos et al., 2013). Intracerebroventricular injection of 24S,25-EC to WT mouse brain embryos *in utero* increased the number of midbrain dopaminergic neurons *in vivo* adding further weight to the hypothesis that the neuro-oxysterol 24S,25-EC promotes dopaminergic neurogenesis (Theofilopoulos et al., 2019). This led to the suggestion that increasing the levels of 24S,25-EC *in vivo* may be a useful strategy to combat the loss of midbrain dopaminergic neurons in Parkinson's disease. Interestingly, the concept of adeno-associated virus (AAV) gene transfer of *CYP46A1* for the treatment of Alzheimer's disease (Burlot et al., 2015), Huntigton's disease (HD) (Boussicault et al., 2016; Kacher et al., 2019) and spinocerebellar ataxias (Nobrega

et al., 2019) has been tested in mouse models of these diseases with success, with the aim of enhancing cholesterol metabolism via oxidation to 24S-HC. Such administration of *CYP46A1* no doubt enhances 24S,25-EC biosynthesis as well, although this was not measured in these studies, and could potentially be used to treat Parkinson's disease also. In the study by Kacher et al using the zQ175 mouse model of HD, *CYP46A1* gene transfer to striatum was found to rectify defects in cholesterol metabolism and alleviate the HD phenotype (Kacher et al., 2019). The expression of the LXR target gene *Apoe* was upregulated and the transport of cholesterol from astrocytes to neurons partially explained the improved phenotype (Kacher et al., 2019). It should be noted that the anti-HIV drug Efavirenz activates CYP46A1 and could provide an alternative route to enhancing 24S-HC and 24S,25-EC formation in brain (Mast et al., 2017).

Studies on the *Lxr* $\alpha^{-/-}$ *Lxr* $\beta^{-/-}$ adult mouse reveal progressive accumulation of lipids in the brain and loss of spinal cord motor neurons and ventral midbrain dopaminergic neurons, indicating further that these receptors are important in brain in both the developing and adult mouse (Wang, Schuster, Hultenby, Zhang, Andersson & Gustafsson, 2002). Surprisingly, studies of the *CYP46A1* tg adult mouse found little change in the mRNA of LXR target genes in brain (Shafaati et al., 2011), this is despite elevation in 24S-HC and 24S,25-EC levels (Shafaati et al., 2011; Theofilopoulos et al., 2019). This may be a consequence of making global measurements where changes in specific brain regions are lost by averaging signal levels in bulk tissue.

Cholestenoic acids are also ligands to LXRs, these include 3β-hydroxycholest-5-en-(25R)26-oic (3β-HCA), 3β-hydroxy-7-oxocholest-5-en-(25R)26-oic (3βH,7O-CA) and 3β,7α-dihydroxycholest-5-en-(25R)26-oic (3β,7α-diHCA) acids, but not its down-stream metabolite 7α-hydroxy-3-oxocholest-4-en-(25R)26-oic (7αH,3O-CA) acids (Figure 3) (Ogundare et al., 2010; Song & Liao, 2000; Theofilopoulos et al., 2014). All of acids are identified in human CSF, with the exception of 3βH,7O-CA which has been found in plasma (Griffiths et al., 2019b; Ogundare et al., 2010; Theofilopoulos et al., 2014), and the two 7α-hydroxy acids have been identified in mouse brain making them neuro-oxysterol-acids (Yutuc et al., 2020). 3β-HCA, 3β,7α-diHCA and 7αH,3O-CA have also been identified in human brain. Remarkably, 3β-HCA is neurotoxic towards motor neurons while 3β,7α-diHCA is protective and 3βH,7O-CA promotes maturation of precursor cells into motor neurons, with each activity mediated by LXRs (Theofilopoulos et al., 2014). Theofilopoulos et al suggested that the loss of motor function in two diseases resulting from inborn errors of metabolism, cerebrotendinous xanthomatosis (CTX, deficiency in CYP27A1) and hereditary spastic paresis type 5 (SPG5, deficiency in CYP7B1), is a consequence of a reduced production of 3β,7α-diHCA in CTX with the additional over production of neurotoxic 3β-HCA in SPG5 providing a double-hit mechanism in the latter disease (see Figure 3 for CYP catalysed reactions) (Theofilopoulos et al., 2014). Interestingly, 3β,7α-diHCA is found to be most abundant in mouse cerebellum but is absent in cerebellum from CTX patients (Yutuc et al., 2020). Cerebellar ataxia, impaired co-ordination of voluntary movements due to underdevelopment of the cerebellum, is a common characteristic of CTX, and may occur in SPG5 also (Bjorkhem, 2013; Clayton, 2011; Salen & Steiner, 2017), linking cholestenoic acids and LXRs to brain development. Besides the cholestenoic acids, (25R)26-hydroxycholesterol ((25R)26-HC, also called by the non-systematic name 27-hydroxycholesterol, 27-HC, note if stereochemistry at C25 is not defined it is assumed to be 25R), a weak LXR agonist (Fu et al., 2001), also accumulates in cerebrospinal fluid of SPG5 patients (Schols et al., 2017; Theofilopoulos et al., 2014) and Hauser et al have suggested that neurotoxic effects of (25R)26-HC are major contributors to the SPG5 phenotype (Hauser et al., 2019). It is noteworthy that neither *Cyp27a1*$^{-/-}$ or *Cyp7b1*$^{-/-}$ mice shows a motor neuron phenotype. A possible explanation for these differences between human and mouse is the prevalence of alternative pathways to produce neuroprotective 3β,7α-diHCA in mouse (Griffiths et al., 2019a; Meljon et al., 2019), or alternatively the presence of lower levels of neurotoxic neuro-oxysterols in mouse than human (Hauser et al., 2019).

Neuro-oxysyerols in the form of 24S-HC and its activation of LXRs has been suggested to be protective against glioblastoma, the most common primary malignant brain tumour in adults (Han et al., 2020). Efavirenz, an antiretroviral medication that crosses the BBB and activates CYP46A1 (Mast et al., 2017), was shown to inhibit glioblastoma growth, the effect being explained by enhanced synthesis of 24S-HC, activation of LXR and inhibition of the cholesterol synthesis pathway by inhibition of SREBP processing (Han et al., 2020). The results reported by Han et al are interesting in a number of regards (Han et al.,

2020):- (i) despite the name, the cellular origin of glioblastoma is unknown, but if glia are the origin of the cancer the effect of 24S-HC must be via a paracrine mechanism as CYP46A1 is expressed in neurons not glia (Lund, Guileyardo & Russell, 1999); (ii) the SREBP studied by Han et al was SREBP-1, not SREBP-2 the dominating transcription factor regulating cholesterol synthesis (Horton, Goldstein & Brown, 2002), 24S-HC was found to reduce nuclear SREBP-1, which is expected (Wang, Muneton, Sjovall, Jovanovic & Griffiths, 2008), but SREBP-1, primarily activates fatty acid not cholesterol synthesis (Horton, Goldstein & Brown, 2002), hence the link to reduced cholesterol synthesis and uptake via LDLR is less clear. Never-the-less the beneficial effects of 24S-HC suggest CYP46A1 as a potential therapeutic target.

Estrogen Receptors

The estrogen receptors α (ERα, NR3A1) and β (ERβ, NR3A2) are both present in rodent brain, although in different locations (Warner & Gustafsson, 2015), as are their classical steroid hormone agonists (Li & Gibbs, 2019) and oxysterol modulators (Griffiths et al., 2019a; Meljon et al., 2019). ERβ has a role in the migration of cortical neurons in the developing brain, where it is detectable at E12.5 (Wang, Andersson, Warner & Gustafsson, 2003), and in adult in the maintenance of serotonergic neurons in the dorsal raphe nucleus, involved in fear, anxiety and depression (Suzuki et al., 2013). ERα is expressed in spinal cord motor neurons where it has a role in protecting neurons against cytokine toxicity (Das, Smith, Gibson, Varma, Ray & Banik, 2011). Both ERα and ERβ are protective against EAE, a mouse model of multiple sclerosis (Spence et al., 2013). The protective effect of ERα is through astrocytes but that of ERβ through microglia (Wu, Tan, Dai, Krishnan, Warner & Gustafsson, 2013). Microglia are the macrophages of the brain, and when activated can damage healthy neurons in the region of infection. ERβ selective agonists dampen the activation of microglia and reduce the proinflammatory potential of invading T-cells (Warner & Gustafsson, 2015; Wu, Tan, Dai, Krishnan, Warner & Gustafsson, 2013).

Besides the ER ligands based on the estradiol skeleton, oxysterols can also act as ligands to both ERα and ERβ (DuSell, Umetani, Shaul, Mangelsdorf & McDonnell, 2008; Umetani et al., 2007). Umetani et al in 2007 and DuSell et al in 2008 both identified 27-HC, presumably (25R)26-HC, as a selective estrogen receptor modulator (SERM) (DuSell, Umetani, Shaul, Mangelsdorf & McDonnell, 2008; Umetani et al., 2007). SERMs are ER ligands whose relative agonist/antagonist activities vary in a cell- and promotor-dependent manner (Wardell, Nelson & McDonnell, 2014). Selectivity is based on the ability of ligands to induce alterations in the ER structure leading to differential recruitment of co-activators and co-repressors. (25R)26-HC induces conformational changes in both ERα and ERβ. Interestingly, (25R)26-HC is a competitive antagonist of ER action in the vasculature (Umetani et al., 2007) but has ER agonist activity in breast cancer cells (DuSell, Umetani, Shaul, Mangelsdorf & McDonnell, 2008). Although most studies of (25R)26-HC and ER have been made in the context of breast cancer (Nelson et al., 2013; Wu et al., 2013) the possibility exists that (25R)26-HC may act as a SERM in the CNS.

In mouse and human brain levels of (25R)26-HC are low (˜ 1 ng/mg) (Griffiths et al., 2019a; Heverin et al., 2004; Meljon et al., 2019), but are elevated in the *Cyp7b1* $^{-/-}$ mouse (˜ 5 ng/mL) (Meljon et al., 2019), and presumably in sufferers of SPG5 where CYP7B1 is deficient and one route of (25R)26-HC metabolism blocked (Figure 3). A raised content of (25R)26-HC in SPG5 brain has not been considered in the context of (25R)26-HC as a SERM, but the role of ERα in the protection of spinal cord motor neurons may be relevant with (25R)26-HC acting as an ERα antagonist in SPG5 where motor neurons are lost. 25-Hydroxycholesterol (25-HC) is also a SERM and is also elevated in *Cyp7b1* $^{-/-}$ mouse brain (Meljon et al., 2019; Simigdala et al., 2016).

Glucocorticoid Receptor

The glucocorticoid receptor (GR, NR3C1) is expressed in brain in both rodent and human, in neurons, glia and vascular epithelial cells (de Kloet, Meijer, de Nicola, de Rijk & Joels, 2018; Tanaka, Fujita, Matsuda, Toku, Sakanaka & Maeda, 1997). The GR is most widely expressed in stress related centres yet within these regions there is differential expression over time. Besides cortisol and corticosterone, the steroid hormone ligands towards GR, this receptor is also activated by 3β,5α-dihydroxycholestan-6-one (3β,5α-diHC-6O, also

called 6-oxocholestan-3β,5α-diol, OCDO, see inset Figure 1), a recently discovered oxysterol derived from cholestane-3β,5α,6β-triol through enzymatic oxidation by hydroxysteroid dehydrogenase 11B2 (HSD11B2) (Voisin et al., 2017), an enzyme expressed in brain (Holmes et al., 2006). Cholestane-3β,5α,6β-triol itself is derived from 5,6-epoxycholesterol (5,6-EC) by the enzyme cholesterol-5,6-epoxide hydrolase (ChEH), an enzyme derived from two subunits DHCR7 (dehydrocholesterol reductase 7) and D8D7I (3β-hydroxystero-delta-8,7-isomerase), however, the enzymatic origin of 5,6-EC has yet to be defined (de Medina, Paillasse, Segala, Poirot & Silvente-Poirot, 2010). In the context of neuro-oxysterols 3β,5α-diHC-6O (OCDO) has been found to be exported from human brain indicating its biosynthesis in this organ (Iuliano et al., 2015).

Retinoic Acid Receptor-Related Orphan Receptors

There are three retinoic acid receptor – related orphan receptors (RORs), RORα (NR1F1), RORβ (NR1F2) and RORγ (NR1F3). RORγ exists as two isoforms RORγ1, or simply RORγ, and RORγ2 also called RORγt. RORγt has a shorter *N*- terminus compared to RORγ but with otherwise has identical domains. RORγt is highly expressed in thymus and is an essential transcription factor for Th17 cell development. Th17 cells are pro-inflammatory T-helper cells ($CD4^+$) that express interleukin 17 (IL-17). Th17 cells are mediators of EAE, the mouse model of multiple sclerosis (Duc, Vigne & Pot, 2019). RORγ is expressed in multiple organs, but not normally in the CNS. However, RORγt-immunoreactive cells have been found in the meninges, the three membranes that envelops the brain and spinal cord, of multiple sclerosis patients, presumably as infiltrating immune cells (Serafini, Rosicarelli, Veroni, Zhou, Reali & Aloisi, 2016). RORα and RORβ are both expressed in brain. RORα is abundant in cerebellum and thalamus, and plays a key role in development, particularly in the regulation of the maturation and survival of Purkinje cells (Jetten & Joo, 2006). RORβ is highly expressed in brain and also the retina (Jetten & Joo, 2006). Oxysterols have been found to bind to the ligand binding domains of both RORα and RORγ (including RORγt), but not as yet RORβ (Duc, Vigne & Pot, 2019).

In 2010 a number of papers were published identifying sterols and oxysterols as ligands to RORα and RORγ (Jin, Martynowski, Zheng, Wada, Xie & Li, 2010; Wang, Kumar, Crumbley, Griffin & Burris, 2010; Wang et al., 2010), these included the archetypical neuro-oxysterol 24S-HC, 24S,25-EC, 20S-hydroxycholesterol (20S-HC), 22R-hydroxycholesterol (22R-HC), 25-HC, 7-oxocholesterol (7-OC), 7α-hydroxycholesterol (7α-HC) and 7β-hydroxycholesterol (7β-HC), all of which have been identified in mouse or human brain (Figure 1) (Yutuc et al., 2020). It is noteworthy that the side-chain oxysterols 20S-HC, 22R-HC 24S-HC, 24S,25-EC and 25-HC are also ligands to the LXRs (Janowski et al., 1999; Janowski, Willy, Devi, Falck & Mangelsdorf, 1996; Lehmann et al., 1997) and with the exception of 20S-HC, which has not been tested, to INSIG (Radhakrishnan, Ikeda, Kwon, Brown & Goldstein, 2007). Cholesterol sulphate, the dominating neuro-sterol sulphate in rat brain (Liu, Sjovall & Griffiths, 2003), has also been shown to bind to the ligand binding domain (LBD) of RORα (Kallen, Schlaeppi, Bitsch, Delhon & Fournier, 2004). RORα is an unusual nuclear receptor in that it is constitutively active in the absence of ligand and despite binding to the LBD of RORα cholesterol sulphate does not appear to affect the transcriptional activity of RORα or RORγ (Wang et al., 2010). In contrast, Wang et al reported that 7α-HC, 7β-HC and also 7-OC to be ligands to RORα and RORγ, and supress the transcriptional activity of the receptors as inverse agonists (Wang et al., 2010). Although CYP7A1 the enzyme required to synthesise 7α-HC and 7-OC is not expressed in brain, 7α-HC, 7-OC and 7β-HC could cross the BBB and be imported from the periphery (Griffiths et al., 2019a; Meaney, Bodin, Diczfalusy & Bjorkhem, 2002), or alternatively, be formed via non-enzymatic reactions initiated by reactive oxygen species (Griffiths & Wang, 2020; Murphy & Johnson, 2008). Interestingly, HSD11B1 is expressed in brain and will convert 7-OC to 7β-HC (Cobice et al., 2013; Mitic et al., 2013) (Figure 1).

24S-HC has also been found to be an inverse agonist of RORα and RORγ supressing the constitutive activity of these receptors (Wang, Kumar, Crumbley, Griffin & Burris, 2010). Interestingly, 24S,25-EC selectively supresses the activity of RORγ (Wang, Kumar, Crumbley, Griffin & Burris, 2010). Conversely, 20S-HC, 22R-HC and 25-HC have been shown to be agonists towards RORγ, promoting the recruitment of co-activators (Jin, Martynowski, Zheng, Wada, Xie & Li, 2010). The crystal structure of the LBD of RORγ with 20S-HC, 22R-HC or 25-HC bound showed the AF-2 helix in a conformation that is permissive for interactions

with coactivator proteins. Importantly, mutations that disrupt the binding of these hydroxycholesterols abolish RORγ transcriptional activity, suggesting a critical role for hydroxycholesterols in activating RORγ (Jin, Martynowski, Zheng, Wada, Xie & Li, 2010). With respect to brain it is interesting to note that CYP46A1, the enzyme that generates 24S-HC, is expressed in Purkinje cells (Lund, Guileyardo & Russell, 1999) and that RORα plays a role in the regulation of the maturation and survival of these cells (Jetten & Joo, 2006). The cerebellum of RORα-deficient mice contains significantly fewer Purkinje cells and exhibits a loss of cerebellar granule cells (Jetten & Joo, 2006). It is tempting to speculate that 24S-HC plays a role in modulating maturation and survival of Purkinje cells via its inverse agonist activity towards RORα. Interestingly, *Cyp7b1* has been reported as a target gene of RORα (Wada et al., 2008). CYP7B1 was first reported in brain and functions as an oxysterol 7α-hydroxylase to side-chain oxysterols including 25-HC and (25R)26-HC (Rose et al., 1997), but not 24S-HC, where CYP39A1 is the 7α-hydroxylase (Li-Hawkins, Lund, Bronson & Russell, 2000).

There is good evidence for the production of 7α,(25R)26-diHC in brain from either imported or *in situ* synthesised (25R)26-HC (Iuliano et al., 2015; Meaney, Lutjohann, Diczfalusy & Bjorkhem, 2000; Yutuc et al., 2020). Based on the publications of Jin et al which indicates that (25R)26-HC activates RORγ and Wang et al that 7α-HC supresses the activity of RORγ it is difficult to predict whether 7α,(25R)26-diHC should be an agonist or inverse agonist towards RORγ (Jin, Martynowski, Zheng, Wada, Xie & Li, 2010; Wang et al., 2010). Soroosh et al have answered this conundrum and shown that 7α,(25R)26-diHC and also 7β,26-dihydroxycholesterol (7β,26-diHC also called 7α,27-diHC) presumably 7β,(25R)26-diHC, are RORγt agonists, reversing the inhibitory effects of the RORγt antagonist, ursolic acid, in cell-based reporter assays (Soroosh et al., 2014). In primary cells both oxysterols were found to enhance the differentiation of IL-17-producing cells in a RORγt dependent manner and Th17 cells were found to produce both oxysterols (Soroosh et al., 2014). Importantly, the pro-inflammatory cytokine IL17-producing $CD4^+$ Th17 cells play a key pathogenic role in multiple sclerosis, and as stated above, RORγt-immunoreactive cells have been found in the meninges of multiple sclerosis patients, presumably from infiltrating immune cells (Serafini, Rosicarelli, Veroni, Zhou, Reali & Aloisi, 2016). While reaction mechanisms for the formation of 7α,(25R)26-diHC are well established, mostly reactions catalysed by CYP27A1 and CYP7B1 or CYP7A1 and CYP27A1 (Griffiths & Wang, 2020), the formation of 7β,(25R)26-diHC is less clear cut, however, it has been identified in plasma from patients suffering from Niemann Pick (NP) disease where 7β-HC and 7-OC are abundant (Griffiths et al., 2019b) and SLOS patients where 7-dehydrocholesterol (7-DHC) is abundant, so mechanisms are available for its formation (Shinkyo, Xu, Tallman, Cheng, Porter & Guengerich, 2011), at least extra-cerebrally (Figures 1 & 2).

G Protein-Coupled Receptors

GPCR183

GPCR183, or EBI2, is a member of the rhodopsin-like subfamily of class A transmembrane spanning GPCRs (Daugvilaite, Arfelt, Benned-Jensen, Sailer & Rosenkilde, 2014). It expressed on the surface of immune cells including T-lymphocytes, monocytes, dendritic cells, astrocytes and innate lymphoid cells (Duc, Vigne & Pot, 2019). GPCR183 is believed to act as a chemoattractant receptor participating in the migration of immune cells down a gradient towards 7α,25-diHC or 7α,(25R)26-diHC (Hannedouche et al., 2011; Liu et al., 2011). Importantly, inflamed white matter of multiple sclerosis patients shows a high expression of GPCR183 compared to non-inflamed white matter (Clottu et al., 2017) and 7α,25-diHC has been found to be increased in spinal cord during EAE development, a mouse model of multiple sclerosis (Wanke et al., 2017), linking GPCR183, its neuro-oxysterol ligand, immune cell migration and multiple sclerosis. Importantly, GPCR183 is expressed by Th17 cells during inflammation and infiltrating cells in multiple sclerosis lesions express GPCR183 (Wanke et al., 2017). Furthermore, expression of cholesterol 25-hydroxylase (CH25H) and CYP7B1, two enzymes involved in 7α,25-diHC biosynthesis (Figure 1), was found to change during the course of EAE with strong up-regulation in spinal cord leading to increased concentration of 7α,25-diHC (Wanke et al., 2017). Remarkably, microglia express CH25H early in EAE, while CYP7B1 is expressed by infiltrating monocytes and lymphocytes (Wanke et al., 2017). This data leads to the conclusion that GPCR183 and

7α,25-diHC play a role in the migration of inflammatory $CD4^+$ T cells into the CNS. 7α,(25R)26-diHC is also a GPCR183 ligand and it is perhaps no coincidence that this molecule is also an agonist towards RORγt.

Smoothened

SMO belongs to the Class F (Frizzled, FZD) of GPCRs and of the 11 members of this class 10 are FZD paralogues, the other is SMO (Kozielewicz et al., 2020). SMO is a seven-pass transmembrane (7TM) protein, essential to the Hh signalling pathway critical in normal animal development, e.g. activation of the Hh pathway is required for the differentiation of neural progenitor cells into motor neuron progenitors, as well as pathological malignancies, including medulloblastoma, the most common paediatric brain tumour.

The Hh signalling pathway is regulated in primary cilia where the transporter protein Patched 1 (PTCH1) and SMO are co-located (Rohatgi, Milenkovic, Corcoran & Scott, 2009). PTCH1 is the receptor for Hh ligands, e.g. sonic hedgehog (SHH), and in the absence of ligand inhibits SMO and the transduction of Hh signals across the plasma membrane (Kong, Siebold & Rohatgi, 2019). When Hh ligands bind to PTCH1 inhibition is released and SMO transmits the Hh signal across the membrane. PTCH1 is structurally related to the sterol transporting protein NPC1 (Lange & Steck, 1998; Pfeffer, 2019), it has 12 transmembrane domains and two sterol binding cavities, and inactivation of PTCH1 by Hh ligands is suggested to allow sterols to accumulate in the cilia sufficiently to activate SMO (Deshpande et al., 2019). The identity of the activating sterol is a point of considerable debate.

Current theories suggest at least two sterol binding sites in the SMO protein. Based on the crystal structure of mouse SMO stabilised in an active state, Deshpande et al suggest one binding site within the 7TM pocket of SMO and a second deep within the extracellular CRD, with a third pocket for the sterol-like antagonist cyclopamine (Deshpande et al., 2019). Deshpande et al argued that both sterol-binding pockets are likely occupied by cholesterol but acknowledged that oxysterols may be alternative occupants of these pockets (Deshpande et al., 2019). Other crystal structures based on inactive SMO failed to identify a 7TM sterol and emphasised cholesterol binding to the CRD as the critical event controlled by PTCH1 (Byrne et al., 2016; Huang et al., 2016). Deshpande et al and Kinnebrew et al have both proposed biophysical models to explain how abundant cholesterol can behave as a signalling molecule (Deshpande et al., 2019; Kinnebrew et al., 2019); there is a precedent for this in the regulation of cholesterol synthesis (and uptake) via the SCAP/SREBP-2 pathway (Goldstein, DeBose-Boyd & Brown, 2006). Deshpande et al proposed a hydrophobic tunnel between TM5 and TM6 of active SMO that opens on the inner leaflet of the membrane bilayer, this may enable sterols in this leaflet to activate SMO without energetically costly membrane desolvation (Deshpande et al., 2019). Activation of SMO via the 7TM-binding sterol may lead to a displacement of TM6, which is further stabilised by sterol binding to the CRD (Deshpande et al., 2019). On the other hand, Kinnebrew et al introduced the concept of "accessible cholesterol" in membrane and cilia in their model (Kinnebrew et al., 2019). They defined three pools of cholesterol, a fixed pool essential to maintain membrane integrity, a sphingomyelin sequestered pool of low accessibility, and an available pool to interact with proteins and be transported to the endoplasmic reticulum (Kinnebrew et al., 2019). The idea of multiple pools of cholesterol was discussed earlier by Radhakrishnan et al and in respect to brain by Dietschy and Turley (Dietschy & Turley, 2004; Radhakrishnan, Anderson & McConnell, 2000). According to Kinnebrew et al's model, the pool of accessible cholesterol in cilia, the subcellular compartment where PTCH1 and SMO are located together, is particularly low. PTCH1 functions in this compartment to selectively transport accessible cholesterol from the cilia to intracellular or extracellular receptors, precluding its binding to the CRD and TM-binding site of SMO (Kinnebrew et al., 2019). Inactivation of PTCH1 will lead to an increase in accessible cholesterol in both leaflets of the ciliary membrane leading to SMO activation through cholesterol binding through both sterol binding sites (Kinnebrew et al., 2019). While Deshpande et al acknowledged that oxysterols may be alternative ligands to these binding sites (Deshpande et al., 2019), Kinnebrew et al argued against this idea based on a CRISPR screen targeting lipid-related genes exploiting the NIH/3T3 cell line (Kinnebrew et al., 2019). While they identified many genes of the cholesterol biosynthesis pathway as positive regulators of the Hh pathway, they failed to find oxysterol synthesising genes to positively regulate Hh signalling (Kinnebrew et al., 2019). However, in the absence of excess cholesterol it is uncertain whether

these cells generate oxysterols under the conditions employed. Interestingly, genes encoding enzymes for sphingomyelin biosynthesis supressed Hh signalling, promoting the concept of a sphingomyelin sequestered inaccessible pool of cholesterol (Kinnebrew et al., 2019).

Despite the study of Kinnebrew et al (Kinnebrew et al., 2019) and the crystal structures showing cholesterol bound to SMO (Byrne et al., 2016; Deshpande et al., 2019; Huang et al., 2016), there is also good evidence that oxysterols activate the Hh signalling pathway, perhaps in a fine-tuning mode akin to their regulation of cholesterol biosynthesis via the INSIG/SCAP/SREBP-2 pathway (Gill, Chow & Brown, 2008). Synthetic oxysterols known to bind to SMO and activate Hh signalling include 20S-HC, 24S-HC, 25-HC, 24S,25-EC, 24-oxocholesterol (24-OC), 7β,(25R)26-diHC, 25-hydroxy-7-oxocholesterol (25H,7O-C), (25R)26-hydroxy-7-oxocholesterol ((25R)26H,7O-C) but not 20R-HC, 7α-HC, 7-OC nor 19-HC (Corcoran & Scott, 2006; Dwyer, Sever, Carlson, Nelson, Beachy & Parhami, 2007; Kim, Meliton, Amantea, Hahn & Parhami, 2007; Myers et al., 2013; Nachtergaele et al., 2012; Nachtergaele et al., 2013; Nedelcu, Liu, Xu, Jao & Salic, 2013; Qi, Liu, Thompson, McDonald, Zhang & Li, 2019; Raleigh et al., 2018). Conversely, 3β,5α-dihydroxycholest-7-en-6-one, an oxysterol derived from 7-DHC and identified in brain of a mouse model of SLOS where 7-DHC is abundant (Xu et al., 2012), binds to SMO and blocks Hh signalling (Sever et al., 2016). Of the above oxysterols 24S,25-EC is abundant in embryonic mouse brain, particularly the ventral midbrain, while 24S-HC and 25-HC are also present during development, but at concentrations about one order of magnitude lower than 24S,25-EC (Theofilopoulos et al., 2013; Wang et al., 2009). In the new-born mouse, 24S,25-EC is still the most abundant oxysterol (Meljon et al., 2012), but in the adult mouse 24S-HC is by far the most abundant oxysterol and 20S-HC is also present but a low level (Meljon et al., 2012; Yutuc et al., 2020). SLOS phenocopies dysregulated Hh signalling (Cooper et al., 2003), however, at least in the new-born mouse the pattern of SMO-activating oxysterols in brain is similar to the WT (Meljon, Watson, Wang, Shackleton & Griffiths, 2013). Of the other oxysterols suggested to activate the Hh pathway through binding to SMO, we have identified 24-OC in brain, but using a derivatisation method where the 24,25-epoxy group isomerises to the 24-oxo so cannot be sure of its exact origin of 24-OC , while 7β,(25R)26-diHC, (25R)26H,7O-C and 25H,7O-C are present in SLOS plasma but essentially absent from control plasma (Meljon et al., 2012; Meljon, Watson, Wang, Shackleton & Griffiths, 2013).

Similar to the situation with cholesterol, there appear to multiple binding sites for oxysterols on SMO. Oxysterols can bind to the same CRD pocket as cholesterol (Byrne et al., 2016) and also within a 7TM pocket (Qi, Liu, Thompson, McDonald, Zhang & Li, 2019; Raleigh et al., 2018). 24S,25-EC appears to bind to and activate SMO via both pockets, while 20S-HC, 7β,(25R)26-diHC, (25R)26H,7O-C and 25H,7O-C act exclusively through the CRD. As discussed above, PTCH1 acts to repress SMO by removing agonist ligands from the plasma membrane in proximity to SMO and exploiting this concept Qi et al were able to extract 24S,25-EC and also 24-OC, 24S-HC and 25-HC from purified PTCH1 (Qi, Liu, Thompson, McDonald, Zhang & Li, 2019). PTCH1 and SMO function together in cilia, and Raleigh et al found 24S,25-EC, 24-OC and 7β,(25R)26-diHC to be enriched in cilia purified from embryonic sea urchin (Raleigh et al., 2018). 7β,(25R)26-diHC and (25R)26H,7O-C are metabolically linked by HSD11B enzymes (Figure 4) (Beck et al., 2019a; Hult et al., 2004; Schweizer, Zurcher, Balazs, Dick & Odermatt, 2004) and both HSD11B1 and HSD11B2 are expressed in brain (Holmes & Seckl, 2006). Interestingly, HSD11B2 is expressed during development brain development (Heine & Rowitch, 2009; Holmes et al., 2006), and will catalyse the reduction of the 7β-hydroxy group to a 7-oxo while HSD11B1 catalyses the reverse oxidation (Beck et al., 2019a; Beck et al., 2019b; Schweizer, Zurcher, Balazs, Dick & Odermatt, 2004). Importantly, *Hsd11b2* is enriched in mouse models of medulloblastoma and *HSD11B2* is enriched in Hh-pathway associated human medulloblastoma (Raleigh et al., 2018). Raleigh et al proposed a mechanism involving HSD11B2 and CYP27A1 by which Hh agonists 7β,(25R)26-diHC and (25R)26H,7O-C could be formed from 7β-HC and, remarkably, pharmacological inhibition of HSD11B2 reduced Hh signalling and tumour growth in mouse medulloblastoma (Raleigh et al., 2018). The contribution of HSD11B2 to oncogenic Hh signalling suggests that oxysterols produced by this enzyme are required for high level pathway activity. Our interpretation of this data is that while (25R)26H,7O-C and 7β,(25R)26-diHC are both agonists to SMO, (25R)26H,7O-C must be more potent. Whether this is a consequence of differences in SMO binding or simply accessibility of ligand to receptor is unclear. While

20S-HC and 24S,25-EC have been identified in brain and are *bona fide* neuro-oxysterols, neither 7β,(25R)26-diHC nor (25R)26H,7O-C have been identified in brain or medulloblastoma, however their precursors 7-OC and 7β-HC have (Griffiths et al., 2019a; Meljon et al., 2019). Importantly, 7O-C and 7β-HC can traverse the blood brain barrier and providing an extracerebral source of precursors for (25R)26-hydroxylation by CYP27A1 (Iuliano et al., 2015).

NMDARs

NMDARs are glutamate-gated ion-channels critical in the regulation of excitatory synaptic function. They are involved in experience-dependent synaptic plasticity and implicated in the cognitive defects of schizophrenia and some forms of autism (Paul et al., 2013). 24S-HC is a positive allosteric modulator (PAM) of NMDARs interacting with the receptors at a site distinct from other allosteric modulators e.g. the PAM pregnenolone sulphate. Even at sub-micromolar concentrations 24S-HC potentiates NMDAR-mediated excitatory post synaptic currents (EPSC) and enhances long term potentiation (LTP) (Paul et al., 2013). While the presence of pregnenolone sulphate in brain is debatable (Liu, Sjovall & Griffiths, 2003), 24S-HC is present in rodent and human brain at concentrations of 10 – 20 ng/mg (25 – 50 μM). Importantly, 24S-HC does not alter membrane currents on its own, indicating that the effect is independent of nuclear receptors e.g. LXR. The synthetic 24-HC analogue, 24,24-dimethylchol-5-en-3β,24-diol (SGE-201), is also a PAM, somewhat more potent than the parent molecule, and it likewise potentiates LTP and, remarkably, reverses behavioural deficits (e.g. spatial working memory) induced by NMDAR channel blockers (Paul et al., 2013). Importantly, the *Cyp46a1*$^{-/-}$ mouse shows a deficiency in hippocampal LTP and a deficit in spatial, associative, and motor learning (Kotti, Ramirez, Pfeiffer, Huber & Russell, 2006). This was explained by Kotti et al to be a consequence of reduced flux of metabolites through the cholesterol biosynthetic pathway and the through the mevalonate pathway towards geranylgeraniol diphosphate (Kotti, Ramirez, Pfeiffer, Huber & Russell, 2006). Administration of geranylgeraniol to hippocampal slices restored LTP to wild type levels indicating that it is a deficit in this molecule, or its diphosphate, which is responsible for the impaired LTP in the*Cyp46a1*$^{-/-}$ mouse (Kotti, Head, McKenna & Russell, 2008; Kotti, Ramirez, Pfeiffer, Huber & Russell, 2006). In light of the data of Paul et al indicating that 24S-HC is a PAM (Paul et al., 2013), it is not unreasonable to speculate that a loss of this molecule in the *Cyp46a1*$^{-/-}$ mouse may also be responsible for some of the deficiency in hippocampal LTP. It is likely that 24S-HC modulates NMDARs in an autocrine or paracrine manner at a distinct site from other PAMs (Paul et al., 2013). Surprisingly, the other side-chain oxysterols 22R-HC and 20S-HC are not modulators of NMDARs, although 25-HC is a weak positive modulator (Linsenbardt et al., 2014; Paul et al., 2013). In-fact, 25-HC is an antagonist of the PAM activity of 24S-HC to NMDARs, but remarkably through a different site (Linsenbardt et al., 2014).

INSIG, SCAP and HMG-CoA Reductase

Sterols and oxysterols regulate cholesterol synthesis by binding to SCAP or INSIG, and via SREBP-2 modulate the expression of the enzymes of the cholesterol biosynthesis pathway (Brown, Radhakrishnan & Goldstein, 2018). At times of cholesterol excess, cholesterol in the endoplasmic reticulum binds to SCAP, which is already in complex with SREBPs, and causes a conformational change in SCAP which results in binding of the SCAP-SREBP complex to the endoplasmic reticulum - anchor protein INSIG. The consequence of this event is retention of SREBP-2 in the endoplasmic reticulum and prevention of its transport by SCAP to the Golgi for processing to its active form as a transcription factor for the enzymes of the cholesterol biosynthesis pathway and for the LDLR (Goldstein, DeBose-Boyd & Brown, 2006; Sun, Seemann, Goldstein & Brown, 2007). Oxysterols also have a role in regulating cholesterol biosynthesis (Kandutsch, Chen & Heiniger, 1978), but in most cases by providing "fine tuning" of this regulation (Gill, Chow & Brown, 2008). The brain is an organ with high oxysterol concentrations (24S-HC 10 – 20 ng/mg cf. cholesterol 10 – 20 μg/mg) and it may be in the CNS that the involvement of oxysterols in regulation cholesterol biosynthesis is most significant (Saeed et al., 2014). Another case when oxysterols may be especially important in regulating cholesterol biosynthesis is in response to infection, both bacterial and virus (Bauman, Bitmansour, McDonald, Thompson, Liang & Russell, 2009; Blanc et al., 2013). Rather than bind to SCAP, side-chain oxysterols i.e. 22R-HC, 24S-HC, 25-HC, (25R)26-HC and 24S,25-EC, bind to INSIG and tether the SCAP-

SREBP-2 complex in the endoplasmic reticulum (Radhakrishnan, Ikeda, Kwon, Brown & Goldstein, 2007). Elegant experiments performed by Radhakrishnan et al exploiting [^{3}H]25-HC and site-directed mutagenesis confirmed that side-chain oxysterols bind to INSIG via a mechanism involving transmembrane helices 3 and 4, which are also involved in INSIG binding to SCAP (Radhakrishnan, Ikeda, Kwon, Brown & Goldstein, 2007). Radhakrishnan et al reported that 19-HC, 4,4-dimethyl cholesterol and lanosterol did not bind to either INSIG or SCAP and did not inhibit the processing of SREBP-2 (Radhakrishnan, Ikeda, Kwon, Brown & Goldstein, 2007).

Conversely, lanosterol, the first sterol in the cholesterol biosynthesis pathway (Figure 2), stimulates the formation of an INSIG-HMG-CoA reductase (HMGCR) complex (Song, Javitt & DeBose-Boyd, 2005) leading to the ubiquitination and degradation of HMGCR, the rate-limiting enzyme of the cholesterol biosynthesis pathway. Cholesterol has no effect on INSIG-HMGCR formation, however 25-HC and other side-chain oxysterols, e.g. (25R)26-HC, 25H,7O-C and 26-hydroxylanosterol (26-HL, also called 27-hydroxylanosterol) and can induce the formation INSIG-HMGCR complex and reductase ubiquitination (Song, Javitt & DeBose-Boyd, 2005). In an early, and largely forgotten study, Axelson et al showed that (25R)26-HC, (25R)26-hydroxycholest-4-en-3-one ((25R)26-HCO), 7α,(25R)26-dihydroxycholest-4-en-3-one (7α,(25R)26-diHCO), 7α,12α,26-trihydroxycholest-4-en-3-one (7α,12α,26-triHCO) were potent suppressors of HMGCR (Axelson, Larsson, Zhang, Shoda & Sjovall, 1995). The first three of these compounds have been found in brain (Griffiths et al., 2019a; Meljon et al., 2019). However, Axelson et al's findings were before the involvement of INSIG in HMGCR degradation was uncovered and a mechanism of HMGCR suppression by oxysterols was not described by these authors (Axelson, Larsson, Zhang, Shoda & Sjovall, 1995). Radhakrishnan et al suggested that it is the oxysterol bound form of INSIG can form a complex with HMGCR, just as it can with SCAP, while lanosterol acts by binding to HMGCR, much like cholesterol binds to SCAP (Radhakrishnan, Ikeda, Kwon, Brown & Goldstein, 2007). Lanosterol is a 4,4-dimethyl sterol and a very recent study has confirmed its ability to stimulate HMGCR degradation and its inability to inhibit SREBP-2 cleavage (Chen et al., 2019; Coates & Brown, 2019). Surprisingly, other 4,4-dimethyl sterols in the cholesterol biosynthesis pathway promoted both HMGCR degradation and inhibited SREBP-2 cleavage (Chen et al., 2019).

Analysis of Neuro-Oxysterols and Neuro-Sterols

Much of our understanding of the biochemistry of neuro-sterols and neuro-oxysterols depends on their reliable detection and quantification in brain tissue, CSF, plasma and in cells of the nervous systems. Both gas chromatography – mass spectrometry (GC-MS) and liquid chromatography (LC)-MS methods have been extensively applied. Gold standard GC-MS methods are based on the classic paper by Dzeletovic et al describing the analysis of oxysterols in plasma (Dzeletovic, Breuer, Lund & Diczfalusy, 1995; Heverin et al., 2004; Schott & Lutjohann, 2015). There are many LC-MS method, with and without the use of derivatisation. A major study using LC-tandem mass spectrometry (MS/MS) has been performed by Russell and colleagues in Dallas in which they analysed over 3,000 serum samples (Stiles, Kozlitina, Thompson, McDonald, King & Russell, 2014). Their analytical method relied on multiple reaction monitoring (MRM) exploiting a tandem quadrupole to achieve maximum sensitivity and with use of multiple isotope labelled standards for quantification (McDonald, Smith, Stiles & Russell, 2012). To achieve high sensitivity others have exploited derivatisation in combination with LC-MS/MS (Honda et al., 2009; Roberg-Larsen et al., 2014; Sidhu et al., 2015; Xu, Korade, Rosado, Liu, Lamberson & Porter, 2011).

Our preference is to use the Girard P (GP) derivatisation reagent in combination with enzymatic oxidation in a methodology called "enzyme-assisted derivatisation for sterol analysis" EADSA (Crick et al., 2015; Griffiths et al., 2013). The method is applicable to any sterol/oxysterol with an oxo group or with a hydroxy group amenable to enzymatic oxidation to a carbonyl. The most commonly used enzyme is cholesterol oxidase which converts 3β-hydroxy-5-ene sterols to 3-oxo-4-enes and 3β-hydroxy-5α-hydrogen structures to 3-ones (Karu et al., 2007). The oxo group is then reacted with the GP hydrazine reagent to give a GP hydrazone which effectively tags the sterol/oxysterol with a positive charge (Figure 5). The consequence of charge-tagging is a major improvement in LC-MS sensitivity. Besides enhancing signal, the charge-tag directs fragmentation in MS/MS or in MS with multistage-fragmentation (MS^n), improving confidence for

identification and allowing the identification of unexpected sterols/oxysterols (Abdel-Khalik et al., 2018; Griffiths et al., 2019a; Ogundare et al., 2010). We find the use of MS^3 in ion-trap mass spectrometers particularly valuable for structural elucidation, where in a first step the GP-derivative $[M]^+$ ion is selected (MS^1), in a second step the $[M]^+$ ion is fragmented (MS^2), the major fragmentation route is the loss of pyridine (Py) from the derivative to give a $[M\text{-}Py]^+$ ion, which is then fragmented in the MS^3 step to reveal structurally informative fragment-ions (Figure 5). To augment MS^3 we like to combine fragmentation information with exact mass information available on Fourier transform hybrid and tribrid mass spectrometers. We have extensively utilised this method to identify and quantify neuro-sterols and neuro-oxysterols in brain and in CSF and to monitor the import and export of these molecules into and out of brain (Iuliano et al., 2015). We have now extended this method to on-surface analysis of tissue slices to localise neuro-oxysterols and neurosterols in mouse brain (Yutuc et al., 2020).

Conclusions

The dominating neuro-oxysterol and neuro-sterol in brain are 24S-hydroxycholesterol and cholesterol. During embryonic development and in the new-born 24S,25-EC is more abundant than 24S-HC in brain. Besides the dominating neuro-oxysterols there are numerous other oxysterols present in brain but at lower concentrations. This is also true for the neuro-sterols where comparatively low-levels of cholesterol precursors are evident. It is however, the most abundant species that appear to be most widely biochemically active. For example, both 24S-HC and 24S,25-EC are ligands to LXR, 24S-HC is an inverse agonist to RORα and RORγ, while 24S,25-EC is an inverse agonist to RORγ only. Both 24S-HC and 24S,25-EC are reported as agonists to SMO and to activate Hh signalling, while 24S-HC is a positive allosteric modulator of the NMDARs while 24S,25-EC has not been tested. In addition, both 24S-HC and 24S,25-EC will bind to INSIG and modulate SREBP-2 regulated cholesterol biosynthesis. Why this redundancy one may ask. The answer may be provided by the *Cyp46a1*$^{-/-}$ mouse which does not synthesise 24S-HC via its usual pathway and 24S-HC is only present in trace amounts in brain, however, other than some learning difficulties the *Cyp46a1*$^{-/-}$ mouse has a mild phenotype. Even in the absence of CYP46A1 in this mouse 24S,25-EC is still the most abundant oxysterol in brain, although at a reduced level compared to the wild-type, and may take on the roles normally performed by the more abundant 24S-HC. Significantly, the *Cyp46A1*$^{-/-}$mouse shows impaired learning and in hippocampal LTP in *vitro* , and while 24S-HC acts as a PAM of the NMDARs and induce LTP, 24S,25-EC has not been reported to do so. So perhaps, the roles of 24S-HC are so important, biology has devised a back-up system using 24S,25-EC.

Besides these two dominating neuro-oxysterols lower abundance relatives may perform specific roles in defined cell populations, at different times and in response to different stimuli.

Figure Legends

Figure 1. Biosynthesis of simple oxysterols. Enzymes are marked in blue. Non-enzymatic oxidation marked in red.

Figure 2. Biosynthesis of 24S,25-EC and other oxysterols from cholesterol precursors. Enzymes are marked in blue. Non-enzymatic oxidation marked in red.

Figure 3. Biosynthesis of cholestenoic acids. Enzymes are marked in blue.

Figure 4. Biosynthesis of SMO ligands from 7-DHC. Enzymes are marked in blue. Non-enzymatic oxidation marked in red. CYP27A1 is the likely catalyst for reactions with broken arrows.

Figure 5. LC-MS(MS^3) analysis of neuro-sterols and neuro-oxysterols after EADSA treatment. (A) Reconstructed ion chromatograms (RICs) showing the most abundant neuro-sterols and neuro-oxysterols extracted from a 400 x 400 μm spot of striatum from a 10 μm thick sagittal section of mouse brain (Yutuc et al., 2020). MS^3 spectra of (B) 24S-HC; and (C) 24S,25-EC from the same brain extract as the chromatograms shown in (A). Note in the EADSA method 24S,25-EC isomerises to 24-OC. (D) RICs showing how the pattern of neuro-oxysterols differ in pooled human plasma (upper panel) and CSF (lower panel) samples. MS^3 spectra

of different oxysterols found in plasma (E) 24S-HC; (F) 25-HC; (G) (25R)26-HC; and (H) 7α-HC. Note the CSF sample was hydrolysed to release oxysterols esterified to fatty acids but the plasma sample was not.

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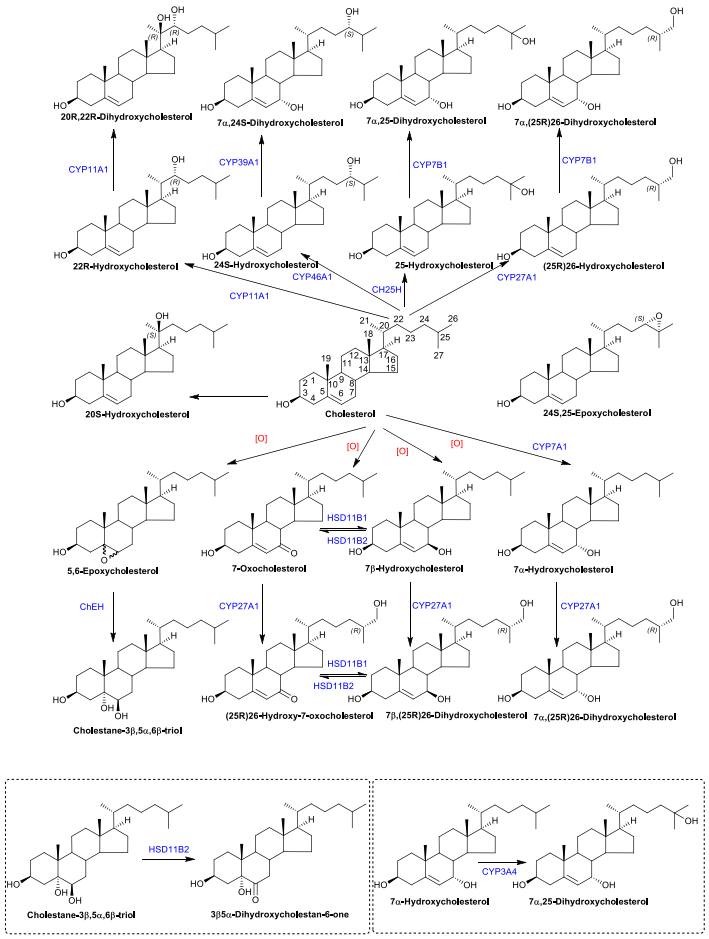
Figure 1



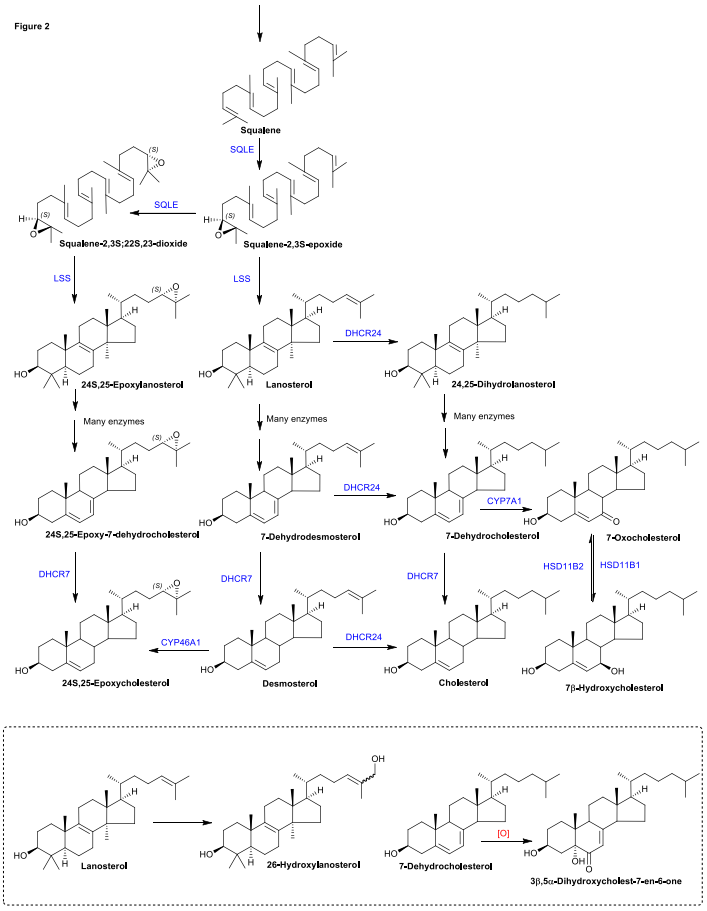


Figure 3

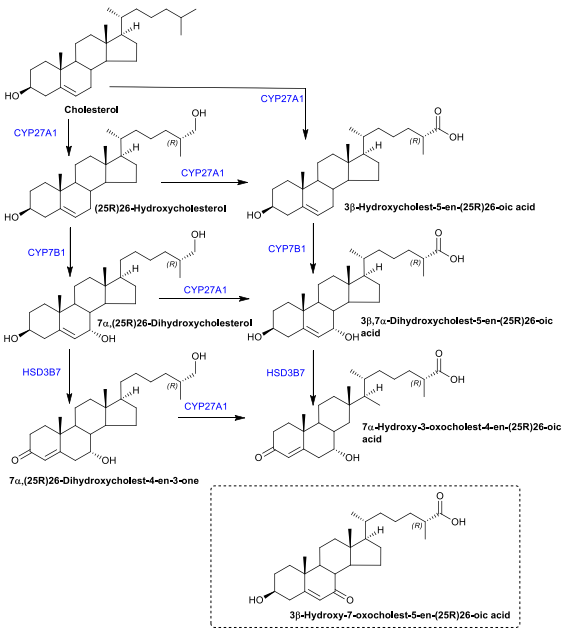


Figure 4