Studies on the Transcriptional and Enzymatic Control of Steroid Metabolism: Regulation by Lysosomal Acid Lipase, 24-Dehydrocholesterol Reductase, and Amyloid Precursor Protein

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ACADEMIC DISSERTATION

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# Table of Contents

Original Publications ........................................................................................................ 5
Abbreviations ..................................................................................................................... 6
Abstract ................................................................................................................................. 8
Review of the Literature ........................................................................................................ 9
  1. Cholesterol .................................................................................................................. 9
     1.1 Whole-body cholesterol metabolism ................................................................. 9
     1.2 Transcriptional regulation of sterol metabolism ................................................... 11
     1.3 Cholesterol biosynthesis .................................................................................... 11
     1.4 Cholesterol uptake and intracellular cholesterol transport .................................. 12
     1.5 Inborn errors of cholesterol metabolism ........................................................... 13
  2. Desmosterol and steroid hormones .............................................................................. 14
     2.1 Desmosterol ......................................................................................................... 14
     2.2 Steroid hormone precursors ............................................................................... 15
     2.3 Metabolism of DHEA ......................................................................................... 16
     2.4 Physiological functions of DHEA ...................................................................... 17
  3. Cholesterol in the brain ............................................................................................... 18
     3.1 Cell types for brain cholesterol processing ......................................................... 18
     3.2 Key proteins for brain cholesterol processing .................................................... 18
     3.3 Developmental regulation of sterols in the brain .................................................. 19
  4. Cholesterol and Alzheimer’s disease .......................................................................... 19
     4.1 Alzheimer’s disease and its animal models ........................................................... 19
     4.2 APP family proteins ........................................................................................... 20
     4.3. APP processing and fragments ......................................................................... 21
     4.4 Links between cholesterol and Alzheimer’s disease ............................................. 23

Aims of the Study ............................................................................................................... 25

Materials and Methods ....................................................................................................... 26
  1. Cell culture and transient transfections (I, II, III) ..................................................... 27
  2. Immunoblotting (I, II, III) ......................................................................................... 27
  3. Radiolabeling of cells (I, III) ..................................................................................... 28
  4. Lipid extraction and analysis (I, II, III) ...................................................................... 29
  5. DNA and cloning and mutagenesis (III) ................................................................. 29
  6. Luciferase assay (III) ............................................................................................... 30
  7. Immunocytochemistry, microscopy, and image analysis (III) .................................... 30
Results and Discussion

1. Role of lysosomal acid lipase in the metabolism of DHEA-FAE-LDL
   1.1 DHEA-FAE-LDL uptake via the LDL receptor
   1.2 Cell metabolites of DHEA-FAE-LDL
   1.3 Lysosomal acid lipase in DHEA-FAE hydrolysis

2. Desmosterol in the developing brain
   2.1 Desmosterol accumulation during brain development
   2.2 Accumulation of desmosterol during brain development is not caused by transcriptional repression of DHCR24
   2.3 Accumulation of desmosterol during brain development may be caused by posttranscriptional repression of DHCR24 by progesterone
   2.4 Accumulating desmosterol may stimulate LXR signaling in the developing brain
   2.5 Accumulating desmosterol may prevent sterol esterification and 24-OHC formation in the developing brain

3. APP and proteolysis products in cholesterol synthesis regulation
   3.1 Role of APP in cholesterol regulation
   3.2 APP fragments in regulating cholesterol synthesis
   3.3 APP regulates cholesterol synthesis via the SREBP2 pathway
   3.4 APP ectodomains regulate cholesterol synthesis via the SREBP2 pathway
   3.5 APP dose effect on cholesterol synthesis in liver cells
   3.6 SREBP2 targets in familial AD patients with APP duplication
   3.7 APP knockdown in primary astrocytes
   3.8 Proposed model for the role of APP in cholesterol balance in the CNS

Conclusions and Future Prospects

Acknowledgement
Original Publications

*Equal contribution


### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>7-DHC</td>
<td>7-dehydrocholesterol</td>
</tr>
<tr>
<td>Aβ</td>
<td>amyloid β</td>
</tr>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette transporter A1</td>
</tr>
<tr>
<td>ABCG1</td>
<td>ATP-binding cassette transporter G1</td>
</tr>
<tr>
<td>ACAT</td>
<td>acyl-Coenzyme A: cholesterol acyltransferase</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AICD</td>
<td>APP intracellular domain</td>
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<td>APLP</td>
<td>amyloid precursor-like protein</td>
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<tr>
<td>ApoA-I</td>
<td>apolipoprotein A-I</td>
</tr>
<tr>
<td>ApoE</td>
<td>apolipoprotein E</td>
</tr>
<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
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<td>APPs</td>
<td>soluble amyloid precursor protein</td>
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<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
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<tr>
<td>CE</td>
<td>cholesteryl ester</td>
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<tr>
<td>CESD</td>
<td>cholesteryl ester storage disease</td>
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<tr>
<td>CETP</td>
<td>cholesteryl ester transfer protein</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COPII</td>
<td>coatamer protein complex II</td>
</tr>
<tr>
<td>DAC</td>
<td>20, 25-diazacholesterol</td>
</tr>
<tr>
<td>DHCR24</td>
<td>24-dehydrocholesterol reductase</td>
</tr>
<tr>
<td>DHEA</td>
<td>dehydroepiandrosterone</td>
</tr>
<tr>
<td>DHEAS</td>
<td>dehydroepiandrosterone sulfate</td>
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<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>FAE</td>
<td>fatty acyl ester</td>
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<tr>
<td>FC</td>
<td>free cholesterol</td>
</tr>
<tr>
<td>FH</td>
<td>familial hypercholesterolemia</td>
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<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HMGR</td>
<td>3-hydroxy-3-methyl-glutaryl-CoA reductase</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>HPTLC</td>
<td>high-performance thin-layer chromatography</td>
</tr>
<tr>
<td>HSD</td>
<td>3β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>IDE</td>
<td>insulin-degrading enzyme</td>
</tr>
<tr>
<td>Insig-1</td>
<td>insulin-induced gene 1</td>
</tr>
<tr>
<td>LAL</td>
<td>lysosomal acid lipase</td>
</tr>
<tr>
<td>LCAT</td>
<td>lecithin-cholesterol acyltransferase</td>
</tr>
<tr>
<td>LD</td>
<td>lipid droplet</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>LDLr</td>
<td>low-density lipoprotein receptor</td>
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<tr>
<td>LRP</td>
<td>low-density lipoprotein receptor-related protein</td>
</tr>
<tr>
<td>LXR</td>
<td>liver X receptor</td>
</tr>
<tr>
<td>NPC</td>
<td>Niemann-Pick disease type C</td>
</tr>
<tr>
<td>NPC1L1</td>
<td>Niemann-Pick C1-like 1</td>
</tr>
<tr>
<td>OHC</td>
<td>hydroxycholesterol</td>
</tr>
<tr>
<td>P450scc</td>
<td>cholesterol side-chain cleavage enzyme</td>
</tr>
<tr>
<td>PM</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>PS</td>
<td>presenilin</td>
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<tr>
<td>RCT</td>
<td>reverse cholesterol transport</td>
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<tr>
<td>S1P</td>
<td>site-1 protease</td>
</tr>
<tr>
<td>SCAP</td>
<td>SREBP cleavage-activating protein</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>---------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>SLOS</td>
<td>Smith-Lemli-Opitz syndrome</td>
</tr>
<tr>
<td>SQLE</td>
<td>squalene epoxidase</td>
</tr>
<tr>
<td>SR-BI</td>
<td>scavenger receptor class B member 1</td>
</tr>
<tr>
<td>SRE</td>
<td>sterol response element</td>
</tr>
<tr>
<td>SREBP</td>
<td>sterol regulatory element-binding proteins</td>
</tr>
<tr>
<td>StAR</td>
<td>steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>STS</td>
<td>steroid sulfatase</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low-density lipoprotein</td>
</tr>
<tr>
<td>WD</td>
<td>Wolman disease</td>
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</table>
Abstract

Cholesterol is an essential structural component of cells. In recent years, many studies have investigated its biochemical and biophysical properties, its metabolism in cells and throughout the body, as well as its pathogenetic roles in diseases. Here we aimed to further explore the metabolic fate of cholesterol, as well as its precursors and metabolites from enzymatic perspectives. The three studies included in this thesis focused on lysosomal acid lipase (LAL), 24-dehydrocholesterol reductase (DHCR24), and amyloid precursor protein (APP). LAL hydrolyzes cholesteryl esters (CE) and generates free cholesterol (FC). DHCR24 converts desmosterol to cholesterol in the last step of the Bloch pathway. APP is the precursor of the pathogenic amyloid β (Aβ) peptide for Alzheimer's disease (AD), and is implicated in the cholesterol metabolism.

We characterized the LAL-mediated degradation of lipoprotein-derived steroid esters in mammalian cells (I). Although the cellular metabolism of low-density lipoprotein (LDL)-carried CE is well studied, the pathway for LDL-borne steroid esters is less well known. We investigated the cellular uptake of dehydroepiandrosterone-fatty acyl ester-LDL (DHEA-FAE-LDL) and its hydrolyzed metabolite pools in cells and in conditioned medium. We also compared the efficacy of DHEA-FAE hydrolysis with that of cholesterol-FAE in HeLa cells and fibroblasts. Our results showed that DHEA-FAE-LDL could be taken up by the LDL receptor (LDLr), after which DHEA-FAE was hydrolyzed partially by LAL and converted into two major metabolites, 5α-androstane-3,6-dione and androstenedione.

We addressed the potential functions of desmosterol, an intermediate precursor of cholesterol, during brain development (II). In the central nervous system (CNS), sterol balance is largely independent of peripheral circulating sterols and has its own regulatory network. Desmosterol transiently accumulates in the developing brain of mammalian species; however, no causal explanation has been established and few consequent effects have been identified. Based on the literature and our own experimental findings, we proposed hypotheses for the cause of desmosterol accumulation and provided evidence for desmosterol regulation in the developing brain by progesterone. Furthermore, we investigated the possible roles of accumulating desmosterol in favoring brain development – desmosterol promotes sterol secretion from astrocytes and maintains an ample supply of active sterols in the developing brain.

We investigated cholesterol metabolism regulation by APP in mammalian cells (III). Although APP has been extensively studied as a pathological factor in Alzheimer's disease (AD), the ubiquity of APP expression in various tissues and the proposed trophic effects of APP on nerve growth suggest a physiological role of APP. In this study, we analyzed cholesterol biosynthesis and uptake regulation by APP and its proteolysis fragments, as well as amyloid precursor-like protein 2 (APLP2), and found that these regulations were mediated via sterol regulatory element-binding protein 2 (SRBEP2), the master protein that governs cholesterol homeostasis by initiating transcription of sterol-related genes. In several cell types (human astrocytic and hepatocytic cells, and human primary fibroblasts), we showed that two APP ectodomains, APPαs and APPβs, acted opposingly in cholesterol synthesis regulation depending on the APP α- vs. β-cleavage, via SREBP2.
Review of the Literature

1. Cholesterol

Cholesterol is an essential building block of the cell membrane and maintains important cellular functions. The molecular structure of cholesterol determines the rigidity and stiffness of lipid bilayers (Figure 1) and directly affects biological events across the cell membrane, e.g., permeabilization of exogenous molecules and signal transduction. Deregulation of cholesterol results in multiple diseases spanning from atherosclerosis to developmental malformations. Therefore, cholesterol content and distribution need to be well maintained at both the cellular and whole-body levels.

Figure 1. Molecular structure of cholesterol.

1.1 Whole-body cholesterol metabolism

1.1.1 Cholesterol absorption

Body cholesterol can be absorbed from the diet by the intestine. Niemann-Pick C1-like 1 (NPC1L1) protein is a crucial mediator in intestinal cholesterol absorption (Altmann et al., 2004). NPC1L1 mRNA is expressed at low levels and mainly in the small intestine. NPC1L1 is a transmembrane protein containing a sterol-sensing domain, expressed in the brush-border membranes of proximal enterocytes. Individuals with heterozygous nonsynonymous NPC1L1 variation have a presumably 50% reduction in sterol uptake and 9% reduction in plasma low-density lipoprotein (LDL)-cholesterol (Ramirez et al., 2011). A cholesterol-lowering drug, ezetimibe, functions as a cholesterol absorption inhibitor by targeting NPC1L1 (Altmann et al., 2004; Garcia-Calvo et al., 2005). Intestinal uptake of dietary cholesterol is followed by cholesterol esterification by acyl-Coenzyme A:cholesterol acyltransferase (ACAT) 2 in the endoplasmic reticulum (ER) in enterocytes and assembled into chylomicron particles for transportation to the liver, cardiac, and skeletal muscle tissues (Nguyen et al., 2012).

1.1.2 Cholesterol balance in peripheral cells and the CNS

Body cholesterol can be redistributed among tissues via the blood circulation. Due to the hydrophobic property of cholesterol, it must associate with lipid-loaded lipoprotein particles to be transported in the circulation. The low-density lipoprotein receptor (LDLr), which was identified in the late 1970s (Goldstein and Brown, 2009), is the receptor for LDL-bound cholesterol uptake and internalization in the peripheral tissues.
Excessive cholesterol in peripheral tissues and macrophages can be delivered back to the liver via reverse cholesterol transport (RCT) and then secreted via the bile into the feces (Glomset, 1968). Alternatively, peripheral cholesterol can also be directly transported through the intestinal wall via transintestinal cholesterol excretion, although this pathway has not been fully elucidated at the molecular level (van der Velde et al., 2010).

RCT is a multistep process largely mediated by high-density lipoprotein (HDL). HDL is produced in the liver and intestine in a lipid-poor discoidal form (pre-β HDL), which can be loaded with free cholesterol (FC) and phospholipids from the peripheral tissues. Apolipoprotein A-I (ApoA-I), a lipoprotein located in the nascent pre-β HDL, interacts with ATP-binding cassette transporter A1 (ABCA1) that promotes cholesterol efflux to the HDL (Brunham et al., 2006), and ATP-binding cassette transporter G1 (ABCG1) that aids in cholesterol transfer. ApoA-I also activates lecithin-cholesterol acyltransferase (LCAT) to esterify FC in the pre-β HDL into cholesteryl ester (CE), thus promoting the formation of mature spherical HDL (HDL₂) (Calabresi and Franceschini, 2010). HDL-borne FC/CE can be taken up by scavenger receptor type-B1 (SR-B1), a cell-surface receptor mainly expressed in the liver and steroidogenic tissues, and utilized in bile acid generation and steroid hormone synthesis, respectively (Kozarsky et al., 1997). HDL binds to SR-B1 and allows delivery of FC and CE to the plasma membrane (PM) without internalization of the HDL particle (Connelly and Williams, 2003). Moreover, HDL₂-bound CE in hepatocytes can be transferred to the LDL and very low-density lipoprotein (VLDL) particles and then enter the LDLr pathway. This step is largely facilitated by cholesteryl ester transfer protein (CETP).

Adipose tissue is the largest cholesterol pool (as much as 25%) in the body; it also acts as a reservoir for triacylglycerides. Adipose tissue is important in preserving excess energy and avoiding lipotoxicity. Cholesterol synthesis rates are low in adipocytes. Most adipocyte cholesterol comes from circulating lipoproteins, e.g. HDL via SR-BI (Dagher et al., 2003) and LDLr-related protein (LRP), oxidized LDL via SR-BI and CD36, another member in the class B scavenger receptor family. Imbalance of cholesterol and other neutral lipids in lipid droplets (LDs), the specialized organelles in the adipose tissue, leads to adipocyte dysfunction, obesity, and insulin resistance (Greenberg et al., 2011).

The central nervous system (CNS) has its own manner of cholesterol synthesis regulation and maintenance, since the blood-brain barrier (BBB) blocks most cholesterol exchange between the CNS and the rest of the body. The CNS needs substantial amount of cholesterol for the important roles cholesterol plays in modulating axon and dendrite outgrowth, neuronal polarity, as well as being a key component in synaptic membranes and myelin that contribute to electrical signaling in neurons (Dietschy and Turley, 2001). Both glial cells and neurons can synthesize cholesterol in situ. However, unlike newborn neurons that produce cholesterol actively for survival, neurons in the postnatal mouse brain have a lower sterol synthesis rate than glial cells, and the latter are considered as the major cholesterol producer (Nieweg et al., 2009). The lipid communication between astrocytes and neurons also occurs via lipoproteins presenting different apolipoproteins: ApoE, which is mainly generated in astrocytes, and ApoJ/ApoD, which are produced by both astrocytes and neurons. ApoE particles are larger than those harboring ApoJ and are required for lipid secretion from astrocytes (Fagan et al., 1999).
1.2 Transcriptional regulation of sterol metabolism

Cholesterol metabolism is one of the most strictly regulated processes in cells, occurring through feedback regulation. The central proteins in this system are sterol regulatory element-binding proteins (SREBPs). In vertebrates, SREBP1 activates fatty acid synthesis, whereas SREBP2 mainly activates cholesterol synthesis and uptake (Horton et al., 2002) by regulating transcription of target genes.

SREBP resides in the ER membrane with both N-terminal basic-helix-loop-helix-leucine zipper (bHLH-Zip) and C-terminus projecting into the cytosol. It binds to SREBP cleavage-activating protein (SCAP), which is retained by insulin-induced gene 1 (Insig-1) under basal conditions with ample cholesterol. In sterol-deprived cells where the ER cholesterol level drops to 5% of the total ER lipids, SCAP binds to coatomer protein complex II (COPII) and leaves the ER via COPII-coated vesicles. SCAP transports SREBP from the ER to the Golgi, where site-1 protease (S1P) first cleaves SREBP in the lumen-projecting hydrophilic loop, and S2P makes the second cleavage within the membrane, generating the free bHLH-Zip domain (mature form of SREBP). Mature SREBP then translocates to the nucleus, where it interacts with the sterol response element (SRE) in target genes, which was first identified in the enhancer region of the LDLr promoter sequence (Sudhof et al., 1987) and initiates transcription of target genes.

Mature SREBP continuously activates gene transcription until a sufficient amount of Insig-1 protein, which is also a transcriptional target of SREBP, has been produced. Insig-1 and SCAP sense the cellular sterol levels by direct binding with oxysterol and cholesterol, respectively, in their sterol-sensing domain, and then trigger Insig-1/SCAP interaction. This leads to a conformational change in the cytoplasmic region of SCAP and dissociates it from the COPII proteins, hence it retains SREBP in the ER (Brown and Goldstein, 2009).

In addition to transcriptional regulation of cholesterol synthesis, cells have also developed a similar feedback system for controlling excessive cholesterol efflux to the environment. Two proteins facilitating the efflux, ABCA1 and ABCG1, are transcriptionally regulated by liver X receptors (LXRs). LXR also helps in suppressing cholesterol uptake by transcriptionally inducing Idol (inducible degrader of the LDLr) expression and triggering LDLr ubiquitination and degradation (Zelcer et al., 2009). LXRs (LXRα and LXRβ) belong to the nuclear receptor superfamily of ligand-activated transcription factors. LXRs use oxysterols as ligands to sense the increased cellular cholesterol level and activate gene expression to protect cells from cholesterol surplus (Janowski et al., 1996). To initiate gene transcription, LXR heterodimerizes with the retinoid X receptor, resulting in a conformational change to recruit nuclear receptor coactivators for transcription activation (Yang et al., 2006).

1.3 Cholesterol biosynthesis

Cholesterol biosynthesis is a complex multistep process involving 35 enzymes (Gaylor, 2002) that are commonly found in the ER (Reinhart et al., 1987). Several of these have been identified as rate-limiting enzymes for the entire synthesis pathway (Figure 2). Squalene epoxidase (SQLE) catalyzes the first oxygenation step in the synthesis pathway; 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) converts HMG-CoA to mevalonic acid; lanosterol demethylase (CYP51), a cytochrome P450 family enzyme, catalyzes the first step after cyclization; 24-dehydrocholesterol reductase (DHCR24) catalyzes the
reduction of the Δ24 double bond in sterol intermediates, converting from desmosterol to cholesterol; and 7-dehydrocholesterol reductase (DHCR7) catalyzes 7-dehydrocholesterol (7-DHC) to cholesterol. Due to its importance in the cholesterol synthesis pathway, HMGCR has been shown to be the target of the cholesterol-lowering drugs, statins (Tobert, 2003). After lanosterol synthesis, the pathway is divided into the Bloch (Bloch, 1965) and Kandutsch-Russell (Kandutsch and Russell, 1960) pathways, which differ at the steps of the Δ24 double bond being reduced. The Δ24 double bond of sterol intermediates in the Bloch pathway can be removed by DHCR24, which converts from the Bloch to the Kandutsch-Russell pathway.

Minor structural differences between cholesterol and its precursors along the biosynthetic pathways, e.g., position of the double bond, may result in considerable alteration of membrane organization and dynamics (Vainio et al., 2006). Therefore, accumulation of cholesterol precursors would lead to serious pathological consequences, as seen in severe inborn diseases due to cholesterol synthesis enzyme deficiencies (Herman, 2003).

Figure 2. Schematic illustration of cholesterol synthesis pathway.

1.4 Cholesterol uptake and intracellular cholesterol transport

Cells take up LDL-associating cholesterol from plasma via LDLr, the process of which has been studied extensively as a classic model for receptor-mediated endocytosis (Goldstein et al., 1985). The LDL receptors cluster in clathrin-coated pits. During external LDL binding, complexes of LDL and the receptor are internalized to the cell via clathrin-coated vesicles. These complexes then enter the endocytic pathway until they reach the lysosome, where the receptors disassociate from the complexes and the CEs become hydrolyzed into FC and fatty acids by lysosomal acid lipase (LAL) at low pH. The disassociated LDL receptors are recycled back to the PM via vesicles budding from the endosomes and ready to be reused for the next binding event.

Niemann-Pick disease type C protein (NPC) 1 and NPC2 are two lysosomal proteins that have been identified as FC transporters. NPC1 is a transmembrane protein with a sterol-sensing domain and is considered as a lipid permease (Davies et al., 2000). NPC2 protein is soluble with a high cholesterol-binding affinity (Ko et al., 2003). X-ray crystallography shows that NPC2 binds cholesterol with its isooyl side chain buried and its 3β-hydroxyl chain exposed, while the N-terminal domain of NPC1 binds cholesterol in the opposite orientation (Kwon et al., 2009). A model of how NPC1 and NPC2 act in tandem in transferring cholesterol has been proposed: NPC2 delivers cholesterol to NPC1 by
direct interaction at their surface patches to allow a ‘hydrophobic handoff’ (Wang et al., 2010).

After being liberated from lyso/endosomes, cholesterol is transported to the ER or PM (Ikonen, 2008). Several players in the cholesterol trafficking en route to the ER have been proposed (Du et al., 2013; Du et al., 2011); however, the machinery of cholesterol transport to the PM was still lacking. Recently, Rab8a, a small GTPase involved in vesicular traffic, and its interaction partners have been identified as key regulators of postendosomal cholesterol transport to the PM, using BODIPY-labeled CE as tracers (Kanerva et al., 2013). The cholesterol released from the lysosomes forms a sterol pool representing the cellular sterol level, which can be sensed by SREBP2-mediated feedback regulation of sterol synthesis. The FC is also subjected to Acyl-CoA:cholesterol acyltransferase (ACAT) for reesterification to maintain the unesterified cholesterol at a constant level.

1.5 Inborn errors of cholesterol metabolism

Familial hypercholesterolemia (FH, OMIM 143890) is an autosomal dominant genetic disorder leading to a deficiency in removing LDL-cholesterol from the circulation. Homozygous FH is rare and found in 1 per million individuals. Heterozygous FH, as the most common inborn error of cholesterol metabolism, has a high incidence of 1:500 among the general population, resulting in 50% risk of coronary artery disease in males and 30% in females by age 60, compared with 13% and 9% in their non-FH relatives, respectively (Stone et al., 1974). The genetic causes of FH are found in mutations of several genes, with LDLr gene mutations as the major phenotype. Over 700 LDLr mutations have been identified in FH patients (Heath et al., 2001); however, these account for only 30-50% of the phenotype in diagnosed patients. In addition to LDLr gene mutations, rare mutations in LDLr ligand ApoB (LDL binding), protease PCSK9 (LDLr protein level) (Abifadel et al., 2003), and autosomal recessive hypercholesterolemia (LDLr internalization) (Garcia et al., 2001) have also been recognized in FH, and more unidentified genes are expected. Statin, the inhibitor of HMGR, has been extensively used in treating both homozygous (Marais et al., 1997b) and heterozygous (Marais et al., 1997a) FH for its effectiveness in reducing LDL-cholesterol.

Cholesterol biosynthesis disorders rising from inborn cholesterol synthesis enzyme defects are usually associated with developmental malformation. The first described, and by far the most common disorder of postsqualene cholesterol biosynthesis, is the Smith-Lemli-Opitz syndrome (SLOS, OMIM 270400), manifesting as decreased plasma cholesterol and increased 7-DHC levels, due to DHCR7 deficiency in the final step of cholesterol synthesis. SLOS has a broad clinical spectrum, from minor physical stigmata to prenatal/neonatal death due to multiple malformations (Porter, 2003). Two other cholesterol biosynthesis disorders, desmosterolosis and lathosterolosis, have been reported in only a few patients, but have features reminiscent of SLOS. Both disorders are deficient in the last steps of cholesterol synthesis, resulting in accumulating plasma desmosterol or lathosterol. In comparison to earlier deficiencies in cholesterol biosynthesis, e.g. congenital hemidysplasia with ichthyosiform erythroderma or nevus and limb defects (CHILD syndrome, OMIM 308050), later deficiencies are clinically more severe (Ikonen, 2006). The severe developmental failure found in these cholesterol biosynthesis disorders could be explained at least partially by the fact that covalent cholesterol modification of Hedgehog is necessary for Hedgehog processing and functioning, which plays a central role in development. SLOS and lathosterolosis display diminished Hedgehog signaling (Cooper et al., 2003).
LAL deficiency (OMIM 278000) causes two distinct autosomal recessive disorders in humans: cholesteryl ester storage disease (CESD) and Wolman disease (WD). LAL is the crucial enzyme for hydrolysis of CE and triacylglycerides and is posttranslationally targeted to lysosomes via the mannose-6-phosphate receptor. Both CESD and WD manifest as accumulation of CE and triglycerides in a variety of tissues, such as liver, spleen, and small intestine. CESD is benign with late onset and may only display hepatomegaly, whereas WD is lethal with infantile onset, exhibiting hepatosplenomegaly, steatorrhea, abdominal distention, and bilateral adrenal calcification (Du et al., 1998). This suggests that there may be other factors accounting for the clinical heterogeneities between CESD and WD. For example, the activities of hepatic acid lipase and neutral lipase are quite unlike in the two diseases (Hoeg et al., 1984).

NPC (OMIM 257220) is a fatal neurodegenerative disorder with approximately 1/150000 birth incidence. Genetic analysis shows that 95% of the cases result from NPC1 gene mutations, with the other 5% from NPC2 mutations. Both NPC1 and NPC2 mutations lead to deficiency in lipid egress from late endosomes and lysosomes (Vance, 2006). In NPC patient fibroblasts, LDL uptake and internalization into late endosomes/lysosomes are not impaired, and although ACAT activity appears normal cholesterol esterification is deficient (Pentchev et al., 1985). Cytoskeletal analyses show massive cholesterol accumulation in late endosomes/lysosomes of NPC cells (Blanchette-Mackie et al., 1988). Consequently, NPC fibroblasts do not respond to LDL-mediated feedback regulation of cholesterol synthesis and LDLr activity, resulting in cellular cholesterol accumulation (Lindenthal et al., 2001).

2. Desmosterol and steroid hormones

2.1 Desmosterol

Desmosterol is an immediate precursor of cholesterol in the Bloch pathway with a Δ24 double-bond difference that can be removed by DHCR24, otherwise their structures are identical (Figure 3). However, cholesterol and desmosterol exhibit considerably different biophysical and functional characteristics. A desmosterol-composed membrane shows much less ordering and insolubility than those of cholesterol (Vainio et al., 2006). Replacing cholesterol with desmosterol leads to a perturbed caveolar structure, although caveolar ligand uptake is only moderately inhibited (Jansen et al., 2008).

*Figure 3. Molecular structure of desmosterol.*
Desmosterol is an important regulator in cholesterol balance; it acts as a ligand of LXR by directly binding to LXRα/β and facilitating recruitment of steroid receptor coactivator 1. The unsaturated side chain of desmosterol is sufficient and the oxysterol side chain is not necessary for desmosterol-induced LXR activation. Desmosterol also suppresses the expression of LDLr and HMGR by reducing SREBP2 processing. The effects of desmosterol on regulating ABCA1 expression via LXR and LDLr expression via SREBP2 are dose-dependent (Yang et al., 2006).

2.2 Steroid hormone precursors

Steroid hormones are important regulators in developmental and physiological processes. Steroids encompass a four-ring structure with 17 carbon atoms, which is inherited from their precursor cholesterol. In LDs where most cholesterol is stored in the ester form, CE can be hydrolyzed by hormone-sensitive lipase during stimulation by adrenocorticotropic hormone. StarD4, a member of the steroidal acute regulatory protein (StAR)-related lipid transfer domain family, transports FC from LDs to the outer mitochondrial membrane. FC is further transported by StAR to the inner mitochondrial membrane where steroidogenesis occurs (Miller and Bose, 2011).

Cells that express cholesterol side-chain cleavage enzyme (P450scc) are steroidogenic; however, the steroidogenic pathway is not identical in the various steroidogenic cell types. Since the activity of some enzymes, especially cytochrome P450 family enzymes, is either lacking, reduced, or enhanced in specific tissues, steroidogenesis in adrenal, gonadal, and brain tissues produces and secretes different levels of steroid hormones (Hanukoglu, 1992).

The first and rate-limiting step of steroidogenesis is the conversion of cholesterol to pregnenolone, which is catalyzed by enzyme P450scc encoded by the CYP11A1 gene (Figure 4). Pregnenolone can be next converted either to progesterone by 3β-hydroxysteroid dehydrogenase (HSD) or to 17α-hydroxypregnenolone by steroid 17α-hydroxylase (P450c17), a cytochrome P450 family member, as is P450scc. In addition to 17α-hydroxylase activity, P450c17 can also serve as a 17,20-lyase catalyzing 17α-hydroxypregnenolone to dehydroepiandrosterone (DHEA). These two independent enzymatic activities of P450c17 also determine the conversion of progesterone to 17α-hydroxyprogesterone and androstenedione. Therefore, P450c17 is considered as an important branch point in steroidogenesis.
2.3 Metabolism of DHEA

DHEA is a major steroid in the circulation and the precursor of androgen and estrogen. DHEA is primarily produced in the adrenal gland and partly in the ovaries (Labrie et al., 2003). Metabolism of DHEA is also dependent on HSDs and cytochrome P450 enzymes (Figure 4). The reductase 17βHSD1 and oxidase 17βHSD2 are responsible for the interconversion of DHEA and androstenediol. The 17βHSD2 also catalyzes the oxidation of testosterone, estradiol, and dihydrotestosterone. The oxidative activity of 17βHSD2 is believed to play a physiological role in protecting tissues from being exposed to excessive active steroid hormones (Peltoketo et al., 1999). DHEA and androstenediol can be converted to androgens (androstenedione and testosterone, respectively) by 3βHSD, and the androgens can then undergo aromatization by P450 aromatase (P450aro) to produce estrogens (estrone and estradiol, respectively). P450aro is ubiquitously expressed in both steroidogenic and nonsteroidogenic tissues, but its expression is diversely regulated in different tissues under different hormone stimulations. For instance, the two-cell theory of human estrogen synthesis suggests that most androstenedione synthesized in ovarian theca cells is converted to estrone by P450aro in granulosa cells (Hickey et al., 1989).

DHEA can exit the steroidogenic pathway by being sulfated in its 3β-hydroxyl group by a sulfotransferase (e.g. SULT2A1), and DHEA sulfate (DHEAS) can be hydrolyzed to DHEA by steroid sulfatase (STS). The major sites for DHEA sulfation are the adrenal gland and liver. In comparison to DHEA, DHEAS usually has a very high concentration in the plasma. Since only free DHEA, but not DHEAS, is a substrate for the 3βHSD, the accumulation of DHEA leads to androgenesis.

DHEA also exists in the form of DHEA-fatty acyl esters (DHEA-FAEs) with nanomolar concentrations in the blood. DHEA is esterified by plasma LCAT, and ~46% of the DHEA-FAE is associated with LDL particles and ~37% with HDL (Lavallee et al., 1996; Roy and Belanger, 1989). DHEA-FAE can be transferred from HDL to VLDL and LDL by a CETP-independent mechanism (Provost et al., 1997). Lipoprotein-associated DHEA-FAE is the major form of DHEA to enter the cells via the receptor-mediated internalization pathway (Leszczynski et al., 1989).
2.4 Physiological functions of DHEA

Many studies have shown that DHEA is a steroid with multiple effects. DHEA and DHEAS are able to reduce inflammation and enhance immunity. In a human study, serum DHEA and DHEAS levels level are inversely correlated with that of interleukin-6 (IL-6), one of the pathogenic factors in inflammatory and age-related diseases (Straub et al., 1998). In aged mice, a DHEAS supplement can prevent and/or reverse the lowered regulation of IL-6 production (Daynes et al., 1993). DHEA also inhibits other inflammatory cytokines, e.g. tumor necrosis factor and natural killer cell cytokine, in various cell types (Iwasaki et al., 2004).

As the precursor of the estrogens and androgens that are implicated in mitogenesis and tumorigenesis, DHEA may play a role in tumor growth, although this is still controversial. A mouse study showed no correlation between DHEAS administration (with consequent high DHEA and DHEAS concentrations) and cancer incidence (Pugh et al., 1999). However, the anticarcinogenic activity of DHEA in inhibiting the cell cycle and in protecting from breast cancer has been shown in a variety of cancer cells (Labrie et al., 2003). In contrast, a large-scale study showed no association between DHEAS/DHEA and breast cancer risk overall, but was positively associated with estrogen/progesterone receptor-positive breast cancer (Tworoger et al., 2006). The conflicting results indicate that other factors, e.g. estrogen level and age, may impact the correlation of DHEA and cancer risk. Since STS frees active estrone and DHEA from their inert sulfate forms, it may be important in inhibiting tumorigenesis (Miller and Auchus, 2011).

DHEA may have a therapeutic effect on diabetes. In a diabetic mouse model, DHEA feeding increased insulin sensitivity and prevented the pathogenic aspects of diabetes, such as hyperglycemia and β-cell necrosis (Coleman et al., 1982). DHEA treatment in an obese mouse model showed that DHEA not only reduces body weight but also decreases serum tumor necrosis factor-α, which plays an important role in insulin resistance. These two independent regulations by DHEA both improved insulin sensitivity (Kimura et al., 1998).

There have been investigations of DHEA as a neurosteroid in the brain of rodents, although evidence in the human brain is still lacking. In the CNS of rat, DHEA is synthesized mostly by astrocytes and moderately by neurons, but not oligodendrocytes. This positively correlates with P450c17 mRNA expression levels in these cells (Zwain and Yen, 1999). DHEA can be found throughout all brain regions without concentration specifications and has multiple functions. One study showed that DHEA and DHEAS could stimulate outgrowth of neurite that respectively becomes axon and dendrites in vitro (Compagnone and Mellon, 1998). DHEA and DHEAS also participate in neuronal protection and survival by protecting hippocampal neurons from glutamate toxicity (Kimonides et al., 1998). These effects could be achieved by DHEA and DHEAS acting as agonists for σ receptors (Monnet et al., 1995) and antagonist for γ-aminobutyric acid A receptors (Majewska et al., 1990).

Due to the declining concentration of DHEA with age, and its protective role in age-related disorders such as cardiovascular disease and immunodeficiency, DHEA is considered as a dietary supplement. DHEA may prevent atherosclerosis (Yamakawa et al., 2009) and improves vascular endothelial and insulin sensitivity (Kawano et al., 2003). However, larger-scale clinical studies are needed to further confirm the beneficial effects and assess the side effects of DHEA supplements.
3. Cholesterol in the brain

3.1 Cell types for brain cholesterol processing

About 23% of the total cholesterol pool in the human body is located in the human brain, although the CNS only accounts for 2% of the body weight (Dietschy and Turley, 2001). Since the BBB prohibits traversal of most lipoprotein-associated cholesterol from the plasma to the brain, cholesterol is actively synthesized de novo in both neuron and glial cells, especially in the newborn brain. In the early stage of brain development, most newly synthesized cholesterol is used for myelin production in the oligodendrocytes, and the rest for cell proliferation. As the brain matures and myelination formation significantly decreases, cholesterol de novo synthesis in the brain also declines to a lower but still measureable level. Using squalene synthase knockout (KO) mice, studies suggested that adult neurons survived without cholesterol synthesis, probably by depending on glia as a cholesterol resource (Funfschilling et al., 2007); while newborn neurons must synthesize cholesterol autonomously (Saito et al., 2009). Comparing the sterol profile in neurons and astrocytes of postnatal rats showed that neurons mainly contain sterols of the Kandutsch-Russell pathway, whereas astrocytes contain sterols of the Bloch pathway. A higher cholesterol synthesis rate is also observed in astrocytes than in neurons: in astrocytes, the majority of newly synthesized sterols are accumulated as cholesterol, while in neurons they are accumulated as lanosterol (Nieweg et al., 2009).

3.2 Key proteins for brain cholesterol processing

In the brain, cholesterol recycling between the glial cells and neurons is mediated by lipoprotein secretion and uptake. In this process, ApoE, the major apolipoprotein in the CNS, and ABCA1 produced in astrocytes are important mediators. ApoE forms cholesterol-enriched HDL-like lipoproteins, and ABCA1 facilitates ApoE transport and lipidation. In ABCA1−/− KO mice, the ApoE level in the cortex and cerebrospinal fluid was reduced, and the size of the ApoE-containing lipoprotein was decreased (Wahrle et al., 2004). Lipoproteins secreted from astrocytes contain not only cholesterol and phospholipids, but also cholesterol precursors, which can be transported to neurons for further conversion to cholesterol.

Neurons take up cholesterol from secreted lipoproteins via LDLr for nerve growth, synapse formation, and neuron repair. Interestingly, CNS-specific ABCA1−/− KO mice showed lower plasma HDL cholesterol levels, reduced brain cholesterol content, and enhanced brain uptake of CE from plasma HDL. It is presumably compensated for by increased cholesterol transport across the BBB through brain capillary endothelial cells, since elevated SR-BI expression in the brain capillaries was seen (Karasinska et al., 2009).

The other two ABC transporter family proteins, ABCG1 and ABCG4, are responsible for removing cholesterol precursors and metabolites via lipoproteins from astrocytes and neurons, respectively (Chen et al., 2013). In the ABCG1−/− or ABCG4−/− mouse brain, sterol levels are normal. However, in the ABCG1−/−/ABCG4−/− double-knockout (dKO) mouse brain, efflux of cholesterol and its precursors to lipoproteins is impaired, cholesterol intermediates in the synthesis pathway are accumulated, and cholesterol synthesis is reduced (Wang et al., 2008). In ABCG1−/−/ABCG4−/− dKO mice brain, 24(S)-, 25-, and 27-hydroxycholesterol (24(S)-, 25- and 27-OHC) are significantly accumulated, suggesting that ABCG1 and ABCG4 may facilitate oxysterol efflux (Bojanic et al., 2010).
Accumulation of desmosterol and oxysterols, which are known as LXR agonists, induces ABCA1 expression and ApoE secretion (Wang et al., 2008).

The brain can secrete excess cholesterol to the peripheral circulation by removing oxidized sterol metabolites, i.e. 24S- and 27-OHC, through the BBB. The oxysterols have higher aqueous solubility than cholesterol in traversing the BBB, due to their polar hydroxyl group in the side chain. Cholesterol 24-hydroxylase (CYP46A1), the enzyme responsible for generating 24S-OHC, is mainly located in the brain, thus most 24S-OHC efflux is from the brain to the circulation. On the other hand, the enzyme for the formation of 27-OHC, sterol 27-hydroxylase (CYP27A1), is present in most organs, therefore 27-OHC is able to traverse bidirectionally through and shows a net flux from the circulation to the brain (Heverin et al., 2005).

3.3 Developmental regulation of sterols in the brain

Brain sterol levels are differentially regulated during the various developmental stages. It has been known since 1960 that there is a sharp accumulation of desmosterol in the early stage of brain development. While other cholesterol precursors only present as 1% of the total sterol, at this stage the desmosterol level may transiently increase to as much as 30% (Fumagalli and Paoletti, 1963). Desmosterol is structurally different from cholesterol, resulting in an alteration in biophysical property and function. The reason for desmosterol accumulation in the developing brain has remained unknown. Whether it is merely a nonfunctional by-product of cholesterol synthesis or regulates brain growth still needs to be elucidated.

Cholesterol is the major sterol in the brain. Maintaining an optimal cholesterol level is crucial for myelination, dendritic and axonal differentiation, as well as synaptic activation in the brain development. Myelin functions as an electrical insulator by extending from the PM of oligodendrocytes and wrapping around the axons. Mutant oligodendrocytes with impaired cholesterol synthesis show reduced cholesterol: protein ratios and hypomyelination, although the myelin architecture is undisturbed and still concentrated with the cholesterol. This indicates that cholesterol is essential for myelin membrane growth and that mutant oligodendrocytes must take up cholesterol from other cell types, e.g. astrocytes, to support myelin synthesis (Saher et al., 2005). Oxysterols are also necessary for brain cell formation. LXR deletion results in decreased dopaminergic neurons and accumulation of radial glial cells at birth, while LXR activation by oxysterols leads to increased DA neurons in mouse embryonic stem cells (Sacchetti et al., 2009).

4. Cholesterol and Alzheimer’s disease

4.1 Alzheimer’s disease and its animal models

Alzheimer’s disease (AD, OMIM 104300) is the most common cause of dementia among elderly people. The major pathological hallmarks of AD are abnormal formation of extracellular amyloid β (Aβ) plaques and intracellular tau-containing neurofibrillary tangles in the brain. There are two histopathologically indistinguishable forms of AD, based on the onset time and genetic factors. The rare familial early-onset form of AD (FAD) commences before 65 years of age, typically in patients in their 40s or 50s, and is caused by mutations in genes encoding amyloid precursor protein (APP), presenilin (PS) 1 and PS2 (Borchelt et al., 1996). The more common sporadic late-onset AD does not
involve mutations in these three genes, but 25% of patients with this form of AD have close relatives with dementia, suggesting that there exist other genetic factors (Bird, 2005). Susceptibility to AD is also APP dose-dependent. Down’s syndrome patients bearing an extra chromosome 21 (trisomy 21), thereby with an additional copy of the APP gene, will ultimately develop an AD-like pathology (Rumble et al., 1989).

Several transgenic (TG) AD mouse models have been generated to understand the pathological mechanism of AD, and most of them aim for accumulated brain Aβ in resemblance of AD. Differentially targeted single-, double-, or triple-TG mouse models have been established. For example, APP23 TG mice expressing human Swedish mutant APP (APPsw) show characteristics of human AD, i.e. prominent plaque deposition with a fibrillar Aβ core and neuronal death in the brain (Calhoun et al., 1998). Double-TG mice coexpressing human wild-type APP and FAD-PS1 mutant (M146L, A246E, or dE9) have been developed and show elevated Aβ42 levels and Aβ42: Aβ40 ratios (Borchelt et al., 1996). Double-TG mice coexpressing APPsw and PS1 mutant (A246E or dE9) show a positive correlation between Aβ42 elevation and the age of amyloid onset (Jankowsky et al., 2004). Coexpression of transforming growth factor-β1 in APP TG mice that develop AD-like pathology accelerates the deposition of Aβ peptide (Wyss-Coray et al., 1997). A triple-TG mouse model harboring PS1M146V, APPsw, and tauP301L provides insights into determining the relationships among Aβ plaque, synaptic dysfunction, and neurofibrillary tangles (Oddo et al., 2003). There are many other AD models that are not listed here, but their contributions to basic and therapeutic research in AD are valuable.

4.2 APP family proteins

The main component of the amyloid plaque is Aβ peptide, which is a 33-43 amino acids peptide derived from APP. The APP gene was first identified and cloned to chromosome 21 in 1987 (Kang et al., 1987). APP is a type I transmembrane glycoprotein that is detectable in both adult and fetal brain and numerous nonneuronal tissues in humans and lower mammals, e.g. adrenal gland, kidney, spleen, heart, and liver (Selkoe et al., 1988). Neuronal APP is much more of a 695 amino acids splicing form, and nonneuronal APP is more of a 751 or 770 residue form (Haass et al., 1991) that contains a Kunitz-type of serine protease inhibitor motif, which the 695 form lacks. There also exist APP orthologs, such as APP-related protein in Caenorhabditis elegans (Daigle and Li, 1993) and APP-like protein in Drosophila (Rosen et al., 1989).

In mammals, APP belongs to a protein family that includes two other members, APLP (amyloid precursor-like protein) 1 and APLP2, which exhibit highly conserved amino acid and domain structure homology of APP. However, only APP can give rise to the formation of senile amyloid plaques, since both APLP proteins lack the Aβ sequence. Despite the sequence similarity among APP family proteins, their presence in tissues and localization in cells are not identical (Kaden et al., 2009). APP and APLP2 are ubiquitously expressed in the brain and peripheral tissues (Slunt et al., 1994), while APLP1 is only detected in the brain. Live cell imaging shows that APP and APLP2 mainly localize in the intracellular compartments, and APLP1 mostly resides in the PM, especially cell-cell contact sites, thus playing a role in cell adhesion. All APP family proteins are able to form homodimers and heterodimers in their ectodomain, although their distinct localizations lead to different degrees of heterodimerization forms. The homodimerization of APP and heterodimerization of APP with APLPs may abolish Aβ generation (Kaden et al., 2008; Kaden et al., 2009).
Several mouse models have been generated to access the importance of APP family proteins in vivo. Mice with a single-KO in each APP family protein are viable. APP+/− mice show reactive gliosis and decreased locomotor activity (Zheng et al., 1995). APLP1+/− mice exhibit a postnatal growth deficit (Heber et al., 2000). APLP2+/− mice are normal and healthy (von Koch et al., 1997). Interestingly, each single-KO doesn’t lead to compensatory upregulation of the other two APP family members (von Koch et al., 1997; Zheng et al., 1995). APLP2+/−/APLP1+/− and APLP2+/−/APP+/− dKO mice die shortly after birth; however, the APLP1+/−/APP+/− mice are viable, and no APLP2 compensation was observed (Heber et al., 2000). This suggests a redundancy between APLP2 and APP or APLP1, and APLP2 performs a key role among the APP family members. As expected, the triple-KO mice show postnatal mortality (Heber et al., 2000).

In other model organisms, APP is found to play several important roles as well. For instance, loss of the APP ortholog results in developmental lethality in C. elegans (Hornsten et al., 2007). In D. melanogaster, the APP ortholog is required for promoting axonal outgrowth, neurite branching, and synapse formation (Shariati and De Strooper, 2013).

**4.3. APP processing and fragments**

APP experiences complex processings in a highly regulated manner and generates several proteolytic fragments to fulfill their various roles in the cell. The APP processing is directly involved in Alzheimer’s pathology, which may provide insights into AD diagnosis and prevention.

Newly synthesized APP undergoes several posttranslational modifications: N-glycosylation in the ER, O-glycosylation and sulfation within the ectodomain in the Golgi, and phosphorylation within the cytoplasmic domain (Lee et al., 2003). Mature APP is then translocated via secretory vesicles to the PM, where the N-terminal ectodomain faces towards the extracellular milieu and the C-terminal domain faces the cytoplasm. The cell-surface APP can be reinternalized via the clathrin-mediated endocytic pathway, dependent on the APP C-terminal YENPTY domain as a signal (Perez et al., 1999). APP can also be covalently modified by Small Ubiquitin-like Modifier, leading to a reduced amount of Aβ aggregates (Zhang and Sarge, 2008).

APP is subjected to proteolytic cleavages during or after the posttranslational modifications in the secretory pathway (Figure 5). APP is constitutively cleaved in a non-amyloidogenic pathway in the PM and Golgi network by α-secretase (ADAM10, a member of A Disintegrin And Metalloproteinase family), producing the soluble amyloid precursor protein (APPs) α ectodomain and C-terminal fragment C83 (Lammich et al., 1999). Alternatively, APP can go through the amyloidogenic pathway in the Golgi and endosomes, where it is cleaved by β-secretase (BACE1) into APPsβ and C99 (Vassar et al., 1999). Both C83 and C99 can be further processed by γ-secretase to generate P3 + APP intracellular domain (AICD) or Aβ + AICD, respectively (Chow et al., 2010). γ-secretase is a multiprotein complex consisting of PS, nicastrin, anterior pharynx-defective 1, and PS enhancer 2 (Kimberly et al., 2003). The two amino acids shift of γ-cleavage in the intramembrane domain of APP results in two Aβ forms, Aβ42 and Aβ40, of which Aβ42 is highly amyloidogenic. Similarly, processing of APLPs also occurs via α-, β-, and γ-secretases as APP (Eggert et al., 2004). The cellular cholesterol level negatively impacts Aβ formation, while the levels of the APP ectodomains remain unchanged (Simons et al., 1998), because low ER cholesterol levels lead to decreased APP β-cleavage (Runz et al., 2002).
Worth mentioning, the γ-cleavage of APP is a regulated intramembrane proteolysis (RIP) process. Other than APP, γ-secretase also mediates cleavage of a number of transmembrane proteins, including Notch, nephrilysin, LRP, and many others (Kopan and Ilagan, 2004). Interestingly, the cleavage of SREBP proteins is also an RIP, although the enzyme responsible for this process is different from γ-secretase (Tomita et al., 1998).

Figure 5. Schematic illustration of APP proteolytic fragments.

The N-terminal APP ectodomains APPsα and APPsβ can be secreted from cells and found in the brain and cerebrospinal fluid (Palmert et al., 1989). Shedding of APP ectodomains can be enhanced by TNF-α (Slack et al., 2001) and statin via the Rho-associated protein kinase pathway (Pedrini et al., 2005). Both APP ectodomains have been recognized in several physiological roles, such as increasing epidermal growth factor (EGF)-induced proliferation of subventricular zone progenitors (Caille et al., 2004) and promoting neurite outgrowth, synaptogenesis, and cell adhesion (Mattson, 1997). APPsα knock-in grossly attenuates deficits of APP KO mice (Ring et al., 2007) and rescues the postnatal lethality of APLP2−/−/APP−/− dKO mice despite deficits in synaptic transmission (Weyer et al., 2011), whereas APPsβ does not (Li et al., 2010). The N-terminal of APPsβ is a death receptor 6 ligand for triggering axonal degeneration via caspase 6 during trophic factor loss (Nikolaev et al., 2009).

Additionally, the C-terminal APP fragment AICD is a transcriptional factor in gene expression regulation. By binding to the nuclear adaptor protein Fe65, AICD is able to translocate to the nucleus and form a multimeric complex with the histone acetyltransferase Tip60 (Cao and Sudhof, 2001). In this way, AICD regulates the transcription of BACE1 and APP to stimulate its own production (von Rotz et al., 2004). AICD also activates glycogen synthase kinase 3β (GSK3β), which catalyzes hyperphosphorylation of tau protein in the formation of neurofibrillary tangles, contributing to AD pathogenesis (Ghosal et al., 2009). Furthermore, AICD represses LRP1 transcription (Liu et al., 2007), although this finding could not be reproduced in another study (Tamboli et al., 2008). Similarly, ICDs derived from APLPs can also be stabilized by Fe65 and translocated to the nucleus. The degradation of the ICDs of APP and APLPs is mediated by insulin-degrading enzyme (IDE) (Walsh et al., 2003).
Interestingly, Aβ not only acts as the pathological factor of AD, but also plays a role in cellular regulations. Aβ inhibits APP expression by decreasing the AβCD level and complex formation with Fe65. Aβ40 inhibits HMGR activity and downregulates cholesterol synthesis, whereas Aβ42 activates sphingomyelinases and reduces the sphingomyelin level (Grimm et al., 2005).

Preclearance of Aβ is especially important in the brain to prevent Aβ aggregation. Transport of Aβ across the BBB may involve different receptors. The receptor for advanced glycation end products takes up Aβ from the periphery to the brain (Deane et al., 2009). On the other hand, LRP1 and VLDLr mediate Aβ efflux to the blood, therefore soluble LRP1 is considered as a plasma Aβ sink that sequesters Aβ for degradation (Zlokovic et al., 2010). Aβ degradation was enabled by several mechanisms. Neprilysin is a key Aβ-degrading metalloprotease (Iwata et al., 2001) that can be transcriptionally regulated by PS and AICD (Pardossi-Piquard et al., 2005). IDE and angiotensin-converting enzyme are also candidate peptidases for Aβ degradation (Carson and Turner, 2002). Furthermore, the level of ApoE lipidation may also determine the degree of Aβ clearance, since delipidated ApoE exhibits a decreased affinity for Aβ peptides (Tokuda et al., 2000).

4.4 Links between cholesterol and Alzheimer’s disease

ApoE is a major cholesterol carrier protein that includes three isoforms: ApoE ε2 (1-5%), ApoE ε3 (50-90%), and ApoE ε4 (5-35%). ApoE ε4 was identified as a risk factor for AD (Corder et al., 1993), and additional ApoE leads to Aβ filament (formed by Aβ lateral aggregation) formation (Ma et al., 1994). Many experimental investigations have been carried out in attempts to explain the isoform-specific effects of ApoE ε4 in AD susceptibility, and they have shown that ApoE ε4 may play multiple roles in the process. For instance, since ApoE ε4 is structurally different from other ApoE isoforms, it interacts with Aβ at a lower affinity, thus clearing Aβ from the brain less efficiently (Kim et al., 2009). ApoE ε4 also stimulates proinflammatory cytokines, while ApoE ε3 suppresses inflammatory responses (Lynch et al., 2003). Moreover, ApoE ε4 increases BBB leakage (Bell et al., 2012), sequesters glutamate receptors, and impairs synaptic activity (Chen et al., 2010), leading to undermined CNS functioning in AD.

Cellular cholesterol distribution and content are closely associated with Aβ generation, which may eventually give rise to AD pathology. β-cleavage of APP occurs predominantly in cholesterol-rich lipid rafts of the cell membrane, and depletion of cholesterol inhibits Aβ generation (Cordy et al., 2003). On the other hand, the non-amyloidogenic α-processing of APP mainly occurs in cholesterol-poor membranes (Kojro et al., 2001). Since cholesterol helps determine the biophysical properties of cellular membranes and membrane-associated protein activity, it is not surprising that it also affects APP processing, especially when α- and β-cleavage are juxtamembrane events and γ-cleavage is intramembrane, not to mention that the γ-secretase complex and the substrate APP itself are both transmembrane proteins. Abnormal cholesterol accumulation in late endosomes greatly enhanced γ-secretase activity and Aβ levels in the NPC mouse brain (Burns et al., 2003). In contrast, low cholesterol levels are found to inhibit β- and γ-secretase independently (Grimm et al., 2008). Treatment with statin, a cholesterol-lowering drug, also strongly reduces the Aβ level in both primary culture and guinea pigs (Fassbender et al., 2001). Recently, a nuclear magnetic resonance study of C99 structure showed that its membrane-buried GXXXG motif, which drives
dimerization of C99, is crucial for cholesterol binding. The C99-cholesterol binding may enhance the γ-cleavage and reduce α-cleavage of C99 (Barrett et al., 2012). Interestingly, an in vivo study using a cholesterol 24-hydroxylase KO / APPswe TG mouse model detected modestly reduced Aβ levels, but not Aβ plaque, in the hippocampus (Halford and Russell, 2009), suggesting a delicate balance between cholesterol levels and AD pathogenesis that needs to be investigated further.

As evidence points towards that cholesterol directing AD pathogenesis, a recent study of AD patient plasma showed significantly decreased desmosterol and desmosterol: cholesterol ratio, compared with healthy elderly patients. This suggests an imbalanced cholesterol metabolism in AD, and plasma desmosterol level to be a biomarker for AD diagnosis (Sato et al., 2011).
**Aims of the Study**

The aim here was to examine the metabolic fate of cholesterol as well as its precursor sterols and metabolite steroid hormone DHEA, and to determine how these are regulated. The individual aims for each study are listed below:

In the first study, we aimed to investigate the uptake, hydrolysis, and metabolism of DHEA-FAE-LDL in cells. Although relatively high concentrations of DHEA-FAE associated with LDL have been found in blood, how DHEA-FAE-LDL is processed in cells was still elusive. Since the processing of cholesterol-FAE-LDL has been exhaustively studied, we compared the characteristic aspects of it, e.g. LDL uptake by LDLr and hydrolysis by lysosomal LAL, with those of DHEA-FAE-LDL to examine their unique kinetics.

The aim of the second study was to explore the cause for the rapid accumulation of desmosterol during brain development and the functions that accumulating desmosterol may serve. Based on literatures that described desmosterol accumulation and addressed its physiological roles, together with our own experimental results, we proposed the potential cause and effects of the accumulating desmosterol in the developing brain.

In the third study, we aimed to define the physiological role of APP and its proteolytic fragments in regulating cholesterol metabolism. APP-derived Aβ and AICD inhibit cholesterol synthesis and related pathways; however, these studies were mostly conducted using mouse models, hence the results may elude the direct impact of APP. In our study, we used cell lines of astrocytic and hepatic origins as well as primary human fibroblasts, investigated the cholesterol synthesis pathway by transiently manipulating the levels of APP and its processing products, dissected the different roles of these products, and provided the underlying regulatory mechanism.
## Materials and Methods

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<tbody>
<tr>
<td>Cell culture</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Cell transfection</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Protein determination</td>
<td>I, II, III</td>
</tr>
<tr>
<td>SDS-PAGE and Western blotting</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Quantification of Western blots</td>
<td>I, III</td>
</tr>
<tr>
<td>[³H]-cholesteryl oleate labeling</td>
<td>I</td>
</tr>
<tr>
<td>Lipid extraction</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Thin-layer chromatography</td>
<td>I, III</td>
</tr>
<tr>
<td>Cholesterol determination</td>
<td>I, III</td>
</tr>
<tr>
<td>DNA cloning</td>
<td>III</td>
</tr>
<tr>
<td>DNA mutagenesis</td>
<td>III</td>
</tr>
<tr>
<td>Nuclear fractionation</td>
<td>III</td>
</tr>
<tr>
<td>Luciferase assay</td>
<td>III</td>
</tr>
<tr>
<td>Fluorescent staining and microscopy</td>
<td>III</td>
</tr>
<tr>
<td>[³H]-acetate labeling</td>
<td>III</td>
</tr>
<tr>
<td>Ag+ high-performance liquid chromatography</td>
<td>III</td>
</tr>
<tr>
<td>High-performance thin-layer chromatography</td>
<td>II, III</td>
</tr>
</tbody>
</table>
1. Cell culture and transient transfections (I, II, III)

Cells were from ATCC except indicated otherwise. HeLa (I) and HepG2 (III) cells were cultured in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and streptomycin, and 2 mM L-glutamine. U251MG cells (III) were cultured in RPMI medium with the same supplements as above. Human control/Wolman fibroblasts (I) were cultured in Eagle’s minimum essential medium (EMEM) with 10% FBS, 10 mM HEPES pH 7.4, and penicillin, streptomycin, L-glutamine as above. Human control fibroblasts and APP<sup>670</sup> patient fibroblasts (provided by Anne Remes, Kuopio University Hospital) derived from a Finnish AD family (II) were cultured in DMEM with 15% FBS and penicillin, streptomycin, L-glutamine as above. For experiments, the cells were also grown in serum-free medium or 5% lipoprotein-deficient serum (LPDS) medium as indicated.

To introduce small interfering RNA (siRNA) oligos (Biomers) and/or plasmid constructs, Lipofectamine 2000 (Invitrogen) was used for HeLa, U251MG, and HepG2 cells according to the manufacturer’s instructions. GL2 siRNA oligos were used as controls in all transfections, except in the luciferase assays where AllStars (Sigma) was used. After a 4-h transfection in complete medium, the cells were washed with phosphate-buffered saline (PBS) and cultivated in serum-free medium for 20 h, unless otherwise indicated in the experiments. To transfect the siRNA oligos in human fibroblasts, Ribocellin (BioCellChallenge) was used according to the manufacturer’s instructions.

2. Immunoblotting (I, II, III)

The cell samples were lysed in 1% NP40 [except for SREBP2 and phospho-GSK3β serine 9 (S9) blots] with protease inhibitors chymostatin, leupeptin, aprotinin, and pepstatin at 25 µg/ml each. The amount of proteins in the lysate was measured by Protein Assay kit (Bio-Rad).

For mature SREBP2 blotting from nuclear fractionation, the cells were treated with 10 µM MG132 for 1 h before harvesting and lysed in sodium dodecyl sulfate (SDS) buffer containing protease inhibitors (Esplenshade et al., 1999). The cell samples were then prepared as described (DeBose-Boyd et al., 1999) with modifications. Cells from duplicate 60-mm dishes were harvested by trypsinizing and pooled. The cells were then suspended in 400 µl of buffer A (10 mM HEPES-KOH at pH 7.4, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM sodium EDTA, 1 mM sodium EGTA, 250 mM sucrose) with protease inhibitors and swollen on ice for 30 min. The swollen cells were passed through a 22.5-gauge needle about 30 times until a clear separation of the intact nucleus from the rest of the cell membranes appeared. The crude cell fractions were centrifuged at 1000-g (3300 rpm for an 8.5-cm radius) at 4 °C for 7 min. The pellet from the 1000-g spin was resuspended in 30 µl of buffer B (20 mM HEPES-KOH at pH 7.6, 2.5% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM sodium EDTA, 1 mM sodium EGTA) with protease inhibitors, rotated at 4 °C for 1 h, and centrifuged at 100 000 g (50 000 rpm) for 30 min at 4 °C in a TLA 100.2 rotor (Beckman). The supernatant from this spin was designated as the nuclear fraction. The supernatant from the 1000-g spin was used to prepare the membrane fraction by centrifuging at 20 000 g (22 000 rpm) for 15 min at 4 °C in a TLA 100.2 rotor. The pellet was dissolved in 30 µl of SDS lysis buffer (10 mM Tris-HCl at pH 6.8, 0.1 M NaCl, 1% SDS, 1 mM EDTA, 1 mM EGTA) with protease inhibitors and used as the membrane fraction. For precursor SREBP2 blotting from the total cell lysates, the samples were lysed in SDS-buffer as above.
For APP ectodomain blotting, the proteins in the conditioned medium were 1:20 concentrated by using Amicon Ultra 30kDa spin column (Millipore) and the concentrates were subjected to analysis.

The proteins were separated on 6-10% SDS-polyacrylamide gel electrophoresis (PAGE) gels, transferred to Hybond-C Extra membrane, stained with Ponceau S (Bio-Rad), blocked with defatted dry milk in Tris-buffered saline (TBS) with 0.1% Tween-20 (Sigma), and immunoblotted with indicated primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies, followed by enhanced chemiluminescence detection. Anti-β-tubulin and anti-Sp3 antibodies were used as loading controls for the total cell lysates and nuclear fraction, respectively.

For phospho-GSK3β (S9) blotting, the cells were lysed in PBS containing 1% SDS, 5 mM EDTA, protease inhibitors as above and phosphatase inhibitors (5 mM activated Na-orthovanadate, 25 mM NaF). The proteins were separated on 10% SDS-PAGE gels, transferred to Hybond-C Extra membranes, stained with ProAct (Amresco), blocked and incubated with anti-phospho-GSK3β (S9) using the Odyssey system (LI-COR). The intensities of the densitometric scanning of specific bands were quantified using ImageJ software (National Institutes of Health, USA).

Table 1. Antibodies and sources.

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<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Study</th>
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<tr>
<td>Mouse anti-LAL</td>
<td>Abcam (ab36597)</td>
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</tr>
<tr>
<td>Mouse anti-actin</td>
<td>Sigma (A4700)</td>
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<tr>
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<td>Invitrogen (R960-25)</td>
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<tr>
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<td>Sigma (T4026)</td>
<td>II, III</td>
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<tr>
<td>Rabbit anti-ABCA1</td>
<td>Novus (NB 400-105)</td>
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<td>(S9)</td>
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<tr>
<td>Goat anti-mouse IgG HRP</td>
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<tr>
<td>Goat anti-rabbit IgG HRP</td>
<td>Bio-Rad (1706515)</td>
<td>I, II, III</td>
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3. Radiolabeling of cells (I, III)

To prepare [³H]-cholesteryl-FAE-LDL, a 40-mCi concentration of [³H]-cholesteryl olate per mg LDL was dried under N₂ flow and redissolved in dimethyl sulfoxide (DMSO) (10% of final volume). The solution was vortexed for 1 min and mixed with LDL for 2 h at 40 °C. The mixture was dialyzed against 5 l dialysis buffer with 150 mM NaCl and 1 mM EDTA, pH 7.4 four times, 12 h each.

To label the HeLa cells and human fibroblasts with [³H]-cholesterol-LDL and [³H]-DHEA-LDL, the cells were grown for 4 days in LPDS medium before the experiments. The [³H]-
cholesterol-LDL or [³H]-DHEA-LDL (50 µg/ml) was added to the medium and pulse-labeled for 6 h for HeLa cells or 8 h for fibroblasts. After washes with PBS, the cells were chased in serum-free medium for 14-24 h (HeLa) or 24-48 h (fibroblasts). To label the U251MG cells with [³H]-acetate, a 20-µCi final concentration of [³H]-acetate was added in serum-free medium for 2-h pulse-labeling at 37 °C, followed by a 1-h chase at 37 °C in the presence of 2.5 mM mevalonate and 10 µM lovastatin. For [³H]-acetate labeling in HepG2 cells, only 15-min pulse-labeling was performed due to its fast turnaround in these cells.

4. Lipid extraction and analysis (I, II, III)

The lipids were extracted from the cell or tissue samples as in (Bligh and Dyer, 1959). The samples were scraped and suspended or homogenized in 2% NaCl, and aliquots were taken for protein determination (Lowry et al., 1951). To extract, 2 ml of methanol and 1 ml of chloroform were added to the 800 µl suspension and vortexed, then centrifuged at 4 °C, 2500 rpm for 10 min to pellet the precipitated proteins. Chloroform and H₂O (1ml each) were added to the upper phase and preceded with vortex and centrifugation. The lower organic phase contained the lipids for further use.

To quantify the radiolabeled sterols, the lipid extracts were resolved with thin-layer chromatography (TLC). The lipids were applied to a silica plate with the corresponding standards and then scraped and counted in the scintillation counter.

To quantify the unlabeled sterol mass, high-performance TLC (HPTLC) was used. The lipids were applied to the silica plate by a CAMAG machine with the corresponding standards. The plate was then stained by a 3% copper sulfate + 8% phosphorous acid solution, and charred at 180 °C for 5 min. The densitometry of the visualized sterols was quantified using ImageJ.

To obtain high resolution of each sterol, the lipids were separated by high-performance liquid chromatography (HPLC). Following the TLC, the lipids were further extracted from the silica and injected into the HPLC machine. The sterols were eluted in the column: the unlabeled sterols were quantified by their peak area compared with the standards, the radiolabeled sterols were collected in scintillation tubes at the appropriate time points according to those of the standards, and the radioactivities were measured in the scintillation counter.

The mobile phase for separating the FC, CE and triacylglycerides was hexane:diethyl ether:acetic acid 80:20:1; the solvent for separation of the cholesterol and other sterols was petroleum ether:diethyl ether:acetic acid 60:40:1.

The total cholesterol content was measured with an Amplex Red kit (Molecular Probes).

5. DNA and cloning and mutagenesis (III)

Wild-type human APP(695)/pCMV and APP(695)M596V/pCMV constructs were from Dr. Dennis Selkoe (Harvard Medical School Center for Neurologic Diseases & Women’s Hospital, USA). APP-KKAA/pcDNA3 was from Dr. Henri Huttunen (University of Helsinki). Human APP-C59/pCDNA4 was from Dr. Frédéric Checler (Institut de Pharmacologie Moléculaire et Cellulaire Centre, National de la Recherche Scientifique, France).
The APP-C83 and APP-C99 complementary DNAs (cDNAs) were cloned from wild-type human APP(695)/pCMV construct into pcDNA3 and pCR3.1 vectors as HindIII-EcoRI and NheI-NotI fragments, respectively.

Point mutagenesis of a cDNA fragment in the expressing vector was achieved using a Phusion Site-Directed Mutagenesis Kit (Thermo). The point mutation constructs of human APP(695)-F615P/pCMV and APP-C99F615P/pCR3.1 were mutagenized from APP(695)/pCMV and APP-C99/pCR3.1, respectively.

6. Luciferase assay (III)

The LDLr promoter-driven Firefly luciferase activity was determined with the Dual-Luciferase Reporter Assay system (Promega). The LDLr-Firefly-Luc and Renilla-Luc plasmids were transfected for 24 h (or cotransfected with siRNAs) and the luciferase activities measured on a luminometer (Promega). The relative LDLr-Firefly luciferase activity was calculated as the ratio of Firefly to Renilla luciferase activity.

7. Immunocytochemistry, microscopy, and image analysis (III)

To fix cells, the cells were incubated in 4% paraformaldehyde (PFA) dissolved in 250 mM HEPES including 100 µM MgCl₂ and 100 µM CaCl₂ for 30 min, followed by quenching in 50 mM NH₄Cl to avoid autofluorescence from the PFA.

For α-early endosomal antigen 1 (α-EEA1) antibody staining, a 5-min incubation in 0.1% Triton X-100 was used to permeabilize the fibroblasts, and a 30-min blocking in 10% FBS at 37 °C was performed next. The cells were then incubated with primary antibody in 10% FBS at 37 °C for 1 h. After extensive washes, the cells were incubated with secondary antibody in 10% FBS at 37 °C for 30 min. The cells were mounted using Mowiol and 1,4, diazobicyclo-(2.2.2) octane (DABCO).

For Dil-LDL uptake, the cells were grown on coverslips in serum-free medium overnight before 10 µg/ml Dil-LDL was added to the HepG2 for 15-min or to the fibroblasts for 90-min incubation at 37 °C. The cells were PFA-fixed and 4',6-diamidino-2-phenylindole (DAPI)-stained for cell number quantification.

The coverslips were examined using an Olympus Provis microscope with a DP71 camera, and the images were acquired by Cell^A software (Olympus). The EEA1 images were analyzed using Image-Pro Plus software (Media Cybernetics) for particle counting and size analysis, and the Dil-LDL images using ImageJ for fluorescence intensity measurement and cell counting.
Results and Discussion

1. Role of lysosomal acid lipase in the metabolism of DHEA-FAE-LDL

1.1 DHEA-FAE-LDL uptake via the LDL receptor

Considering that the amount of inert DHEA-FAE is ~50% of that of active free DHEA and ~50% of DHEA-FAE is associated with LDL particles in the circulation, it is important to understand how cells take up these circulating DHEA-FAE-LDLs and utilize them to produce physiologically active steroids, especially when the DHEA-FAE proportion increases with age.

To study the cellular metabolism of DHEA-FAE-LDL, we first investigated the route by which it enters cells. It has long been known that LDL receptor mediates the cellular uptake of cholesteryl-FAE-LDL; therefore, we tested whether LDL receptor plays a role in DHEA-FAE-LDL internalization. HeLa cells were incubated with [3H]-DHEA-FAE-LDL in the absence or presence of a 50-fold excess of unlabeled LDL for 6 h, and the cells were harvested afterwards to analyze the incorporated radioactivity. We observed a blunted [3H]-DHEA-FAE-LDL uptake when excess cold LDL was presented, since a significant radioactivity reduction was shown in cells with unlabeled LDL incubation compared with the cells without unlabeled LDL incubation (I, Figure 2A). This indicated that the DHEA-FAE-LDL uptake was mediated by LDL receptor or LDL receptor-related proteins.

1.2 Cell metabolites of DHEA-FAE-LDL

To understand the metabolic fate of the internalized DHEA-FAE-LDL, we analyzed the [3H]-DHEA and [3H]-DHEA-FAE contents in both cells and medium. The cells were incubated with [3H]-DHEA-FAE-LDL for 6 h and chased for 48 h, after which the lipids were extracted from the cells and medium. The lipids were then resolved using a Sephadex LH-20 column and the solvent fractions were collected for analysis. In the fractions comigrating with DHEA-linoleate standard, we detected [3H] labels only in the cell fractions but not in the medium fractions, indicating that [3H]-DHEA-FAE was taken up by the cells. We next found that most of the [3H] label in the medium corresponded to the standards of free DHEA and other metabolites (I, Figure 2B). This suggested that part of the incorporated [3H]-DHEA-FAE had been hydrolyzed, metabolized, and effectively secreted into medium.

We then used TLC to identify the metabolites. We resolved lipids that were previously extracted from cells and medium as (I, Figure 2B) by one-dimensional TLC, and detected the lipids with a cyclone storage phosphor system. The cellular radioactivity comigrated with the [3H]-DHEA-linoleate standard, while medium radioactivity partially comigrated with the [3H]-DHEA standard and partially migrated more rapidly (I, Figure 3), in agreement with the results from fractionation analysis.

To further identify the unknown metabolites that had appeared in the medium, we used two-dimensional TLC and several DHEA metabolite standards: 4-adione, 5α-adione, 5α-androstan-3α-ol-17-one, 3β,5α-adiol-17α, 5α-dihydrotestosterone (5α-DHT), 3α,5α-adiol, 3β,5α-adiol, testosterone, and 5α-androstan-3β-ol-17-one as reference steroids. The 5α-adione (mostly), DHEA, DHEA-FAE, and 4-adione were the only steroids presenting in the medium (I, Figure 4), although the possibility that small amounts of other DHEA metabolites were not detectable using this method or degraded during the
chase time could not be excluded. Moreover, since steroidogenic enzyme profiles may be cell type-specific, different metabolites and contents would be expected in other cell types after DHEA-FAE uptake.

1.3 Lysosomal acid lipase in DHEA-FAE hydrolysis

To determine how DHEA-FAE-LDL is metabolized in cells, we studied the role of LAL in this process, since it is the enzyme that is responsible for the hydrolysis of cholesteryl-FAE-LDL. We transiently depleted LAL by siRNAs in HeLa cells (I, Figure 5A) and analyzed the [3H]-cholesterol-FAE-LDL hydrolysis. As expected, the hydrolysis of [3H]-cholesterol-FAE-LDL was significantly inhibited in LAL-depleted cells compared with the control cells (I, Figure 5B). When the [3H]-DHEA-FAE-LDL was analyzed in the same setup, we found a different pattern from what was observed in the [3H]-cholesterol-FAE-LDL (I, Figure 5B). First, there was significantly more [3H]-DHEA-FAE than [3H]-cholesterol-FAE remaining in the control cells, indicating different kinetics for the hydrolysis of the two FAEs. Second, LAL depletion had a more modest effect on hydrolyzing the [3H]-DHEA-FAE than the [3H]-cholesterol-FAE, suggesting that LAL was not the only enzyme participating in the hydrolysis of [3H]-DHEA.

We also used Wolman cells to investigate the contribution of LAL in the hydrolysis of [3H]-DHEA-FAE and [3H]-cholesterol-FAE. The [3H]-cholesterol-FAE hydrolysis was abolished in these cells, as expected, since CE hydrolysis is LAL-dependent; however, a significant level of [3H]-DHEA-FAE hydrolysis was still observed despite the absence of LAL activity in the Wolman cells. However, the hydrolysis of [3H]-DHEA-FAE was less efficient in the Wolman cells than in the control fibroblasts, implying that LAL played a partial role in this process (I, Figure 6B).

Together, this study characterized the metabolic processing of DHEA-FAE-LDL in the aspects of uptake, hydrolysis, and metabolite secretion (Figure 6). By cellular hydrolysis, the inert DHEA-FAE converts to biologically active DHEA and further to other steroid metabolites. We showed that LAL is not the only enzyme for DHEA-FAE hydrolysis, suggesting the existence of other lipases, such as the hormone-sensitive lipase that mediates E2-FAE hydrolysis, which may constitute the major activity in DHEA-FAE hydrolysis.

Figure 6. Schematic illustration of DHEA-FAE-LDL and cholesteryl-FAE-LDL uptake and hydrolysis in the lysosomes. The endocytic membrane trafficking routes of the LDLs and the exocytic pathway of the hydrolytic products and metabolites have been simplified.
2. Desmosterol in the developing brain

2.1 Desmosterol accumulation during brain development

In the developing mouse brain, desmosterol begins accumulating during embryonic day 14 until postnatal day 10 and peaks in the first postnatal week (II, Figure 1B). Compared with desmosterol, other sterols do not show similar substantial accumulations during this period (II, Figure 1C), and the total sterol content rises remarkably (II, Figure 1D). Since this time window corresponds with the period in which neuron growth and synapse formation actively commence, desmosterol may play a special role in brain development.

2.2 Accumulation of desmosterol during brain development is not caused by transcriptional repression of DHCR24

To investigate the mechanism that regulates desmosterol accumulation during brain development, we analyzed the transcriptional regulation of three important enzymes in the cholesterol biosynthesis pathway: DHCR24, which converts desmosterol to cholesterol; SQLE, a rate limiting enzyme in prelanoster synthesis; and DHCR7, which removes the double bond from 7-DHC, an immediate precursor of cholesterol. We expected to see a decrease in the mRNA level of DHCR24 if it accounted for desmosterol accumulation in the developing brain. However, reverse transcription-polymerase chain reaction (RT-PCR) showed that the DHCR24 mRNA level remained flat at the highest level until P3, overlapping with desmosterol accumulation (II, Figure 2). From P3 to P12, the DHCR24 mRNA level decreased, while the desmosterol level also fell. This suggests that transcriptional regulation of DHCR24 is not the reason for desmosterol accumulation in the developing brain. Moreover, changes in the DHCR24 mRNA profile were very similar to those in SQLE and DHCR7 (II, Figure 2), suggesting that DHCR24 is not regulated differentially from other cholesterol synthesis enzymes. Therefore, instead of repressing the DHCR24 level to cause desmosterol accumulation during brain development, it is more likely that desmosterol plays a role in feedback regulation of DHCR24 expression.

2.3 Accumulation of desmosterol during brain development may be caused by posttranscriptional repression of DHCR24 by progesterone

We next investigated whether the posttranslational regulation of DHCR24 activity affected the desmosterol accumulation. Progesterone and pregnenolone are present in the developing brain (Ibanez et al., 2003). Moreover, they suppress Δ24 reduction and induce desmosterol accumulation in vitro (Lindenthal et al., 2001; Panini et al., 1987). Therefore, we analyzed whether progesterone would inhibit DHCR24 activity via post-translational regulation.

In this study, we used a Chinese hamster ovary (CHO) cell line stably expressing DHCR24. Progesterone incubation did not increase the DHCR24 protein level in these cells or in control CHO cells (II, Figure 3). By pulse-labeling the control CHO cells with [14C]-acetate in the presence of progesterone and analyzing the subsequent sterol metabolic products in the cell, we found that progesterone caused an accumulation of [14C]-desmosterol and a decrease in [14C]-cholesterol. In CHO cells stably expressing DHCR24, however, we observed that the [14C]-desmosterol accumulation during progesterone treatment was attenuated, compared with that in control CHO cells, and
only achieved a similar degree of [14C]-desmosterol accumulation at higher progesterone concentrations (II, Figure 3). These results indicated that progesterone induces desmosterol accumulation via DHCR24; however, this induction is not by reducing the DHCR24 protein level, but most probably by posttranscriptionally inhibiting DHCR24 enzymatic activity.

2.4 Accumulating desmosterol may stimulate LXR signaling in the developing brain

LXR mediates sterol secretion that is essential in the developing brain. Oxysterols were previously believed to be the only LXR regulators; recently, desmosterol was identified as another LXR stimulator in vitro (Yang et al., 2006). We tested the possibility that accumulation of desmosterol may regulate LXR target genes in the developing brain.

We treated U251MG human glioma cells with a DHCR24 inhibitor 20,25-diazacholesterol (DAC) and cultured the cells in LPDS medium to prevent exogenous cholesterol uptake. In 2 days, desmosterol accumulated to 40% of the total sterol. We analyzed the protein level of the LXR target gene ABCA1 in these cells by Western blotting and found that ABCA1 level was significantly increased during DHCR24 inhibition, suggesting that LXR signaling was activated by desmosterol (II, Figure S1A).

Since LXR regulates genes that are involved in sterol secretion, we next studied whether desmosterol accumulation would promote sterol efflux. We analyzed the cellular sterols in control vs. DAC-treated U251MG cells and sterols that were secreted in the medium. We observed an increase in sterols in the medium of DAC-treated cells (II, Figure S1B). These results together suggested that desmosterol accumulation stimulates LXR signaling and subsequently enhances sterol secretion in astrocytic cells. This is in line with the result from a previous study that desmosterol-loaded primary astrocytes exhibited a marked increase in both protein and mRNA levels of ABCA1 (Wang et al., 2008).

2.5 Accumulating desmosterol may prevent sterol esterification and 24-OHC formation in the developing brain

In analyzing the sterol profile during desmosterol accumulation induced by DAC, we also observed a decrease in sterol ester levels in DAC-treated cells (II, Figure 1B), indicating that desmosterol accumulation inhibits sterol esterification. This was evidenced as the desmosterol accumulation in the developing mouse brain, which coincided with decreased sterol ester levels (II, Figure 1D). By maintaining sterols in the active and functional unesterified form, desmosterol could be important for brain activities in the developing brain.

On the other hand, desmosterol accumulation could be beneficial by preventing 24-OHC formation and subsequent sterol egression from the CNS during brain development. Since desmosterol cannot be hydrolyzed as cholesterol at C24 to generate 24-OHC, which passes through the BBB (Ohyama et al., 2006), the brain could effectively utilize de novo- synthesized sterols by accumulating sterols as desmosterol instead of cholesterol.
3. APP and proteolysis products in cholesterol synthesis regulation

Several studies have pointed to the connection between APP and cholesterol homeostasis, especially in attempts to understand the role of cholesterol in AD pathogenesis using AD mouse models. In this study, we aimed to address the ubiquitous physiological role of APP and its proteolytic fragments in regulating cholesterol balance, using human astrocytic (U251MG), hepatic (HepG2), and fibroblastic cells. Astrocytes are considered to be important cholesterol-producing cells in the CNS, hepatocytes are key cholesterol-synthesizing cells at the whole-body level, and fibroblasts are major cholesterol-receiving cells via LDL uptake.

3.1 Role of APP in cholesterol regulation

First, we studied cholesterol synthesis in cells with transient APP depletion. APP has a short half-life measured in hours. In U251MG astrocytoma cells transfected with APP siRNA oligos for 6 h, 12 h and 24 h, we observed APP depletion by ~50% in 6 h and ~90% in 24 h (III, Figure 1A). APLP2 depletion in U251MG cells could also be achieved in 24 h by siRNA oligo transfection (III, Figure S4A). Interestingly, APP and APLP2 compensated mutually in their protein levels when one was depleted for a prolonged time, i.e. 3 days (III, Figure S4D). This implies that the two APP family proteins may serve similar functions, which is in agreement with (Shariati and De Strooper, 2013). Therefore, the transient approach is more suitable for studying the direct role of APP family proteins than using mouse models that have been heavily used in AD research.

In cells with APP depletion for 24 h or less, the cholesterol level was reduced by 10%, independent of serum conditions (III, Figure 1B). In cells cultured under serum-deprived conditions in which cellular cholesterol level relies on de novo synthesis, we observed that [3H]-cholesterol synthesized from [3H]-acetate was also substantially decreased (III, Figure 1C). The same downregulation in cholesterol synthesis was also observed in APLP2-depleted cells (III, Figure S4B).

To prove that APP depletion-induced down-regulation in cholesterol synthesis is specific, we introduced wild-type APP (695) to APP-depleted U251MG cells. Bringing back APP to the cells resulted in a recovery in cholesterol de novo synthesis, confirming the direct role of APP in cholesterol regulation (III, Figure 1E).

Interestingly, a recent study showed that APP directly interacts at its GXXXG motif with SREBP1 in the Golgi, prohibiting the S2P processing of SREBP1 and consequent HMGR expression and cholesterol synthesis in neurons, but not in astrocytes (Pierrot et al., 2013). Since SREBP2, instead of SREBP1, is the major regulator of cholesterol metabolism (Horton et al., 2002), it would be interesting to examine whether APP is able to bind SREBP2 and affect cholesterol synthesis as well. Because the GXXXG motif is also the binding site of APP with cholesterol (Barrett et al., 2012), cholesterol may facilitate the APP-SREBP interaction, resulting in reduced SREBP processing and consequently decreased cholesterol synthesis in conditions with abundant cholesterol. Future study would be beneficial to test this hypothesis.

3.2 APP fragments in regulating cholesterol synthesis

APP undergoes a complicated processing in cells (III, Figure 1D); thus, our observation of the regulatory role for APP in regulating cholesterol synthesis needed to be
elucidated further in terms of APP-processing products. We dissected the role of APP fragments, using the cholesterol synthesis rescue assay as readout.

We obtained the point-mutant APP construct APP-M596V, which is deficient in β-processing and, therefore, generates C83 and APPα (III, Figure 1F). We generated another point-mutant APP construct, APP-F615P, which predominantly undergoes β-cleavage and consequently produces C99 and APPβ (III, Figure 1F). In APP-depleted U251MG cells, overexpression of APP-M596V rescued cholesterol synthesis to the same level as did wild-type APP, while APP-F615P only rescued to a lesser extent (III, Figure 1E). Interestingly, overexpression of an ER-retention APP construct, APP-KKAA, significantly inhibited cholesterol synthesis (III, Figure 1E). Due to the dilylsine ER retrieval mutation in the APP C-terminal, APP-KKAA does not reach the trans-Golgi/PM where most α-cleavage occurs (Skovronskey et al., 1998). This suggests that products from both α- vs. β-cleavage, i.e. C83 and APPα vs. C99 and APPβ, contribute to the differentially regulated cholesterol synthesis.

Since Aβ40 has been shown to inhibit cholesterol synthesis (Grimm et al., 2005), we measured Aβ levels in the media from the conditions above. ELISA analysis of Aβ40 and Aβ42 levels in the conditioned media did not show a corresponding pattern as in the cholesterol synthesis assay (III, Figure S1A), suggesting that both Aβ40 and Aβ42 did not account for the differential regulations by APP-M596V and APP-F615P.

We next tested APP C-terminal intracellular fragments in cholesterol synthesis rescue assay. All C83, C99 and C59 (AICD) restored the synthesis in APP-depleted cells (III, Figure 1G). Because C99 can be a substrate for α-secretase to generate C83 (Jager et al., 2009), we generated a C99-F615P mutant construct that escapes from α-cleavage. C99-F615P overexpression showed no difference from other APP C-terminal fragments (III; Figure 1G). This indicates that all APP C-terminal fragments, no matter whether they are processed by α- or β-secretase, are capable of rescuing cholesterol synthesis. Therefore, the shared proteolytic end product C59/AICD in C83 and C99 may account for this activity.

### 3.3 APP regulates cholesterol synthesis via the SREBP2 pathway

We next investigated the mechanism of APP-mediated cholesterol regulation by examining three cholesterol-specific genes: HMGCR, CYP51 and LDLR. HMGCR and CYP51 are rate-limiting enzymes in the cholesterol synthesis pathway, and LDLR is responsible for cellular uptake of exogenous cholesterol. Due to the importance of HMGCR, CYP51, and LDLR in cholesterol balance, we analyzed their mRNA and protein levels in U251MG cells with 24-h APP depletion. The transcript levels all plunged (III, Figure 2A) and the protein levels were also reduced accordingly (III, Figure 2B). Since these three genes are transcriptionally regulated by SREBP2 at their SRE, we analyzed the SRE activity, using an LDLR promoter-driven luciferase reporter construct. We found decreased SRE activity in APP-depleted cells (III, Figure 2C); similar reduction was also seen in APLP2-depleted cells (III, Figure S4C). This was due to downregulation of SREBP2 activity, since we observed a decrease in mature SREBP2 protein level in the nuclear fraction of APP-depleted cells (III, Figure 2D). In addition, the SREBP2 precursor level was also reduced (III, Figure 2D).

Experimental results in NPC1-depleted U251MG cells further argued for an inhibitory role of the APP β-processing products in regulating cholesterol synthesis via SREBP2. NPC1 depletion in U251MG cells showed increased cholesterol synthesis (III, Figure 2E),
as expected due to an endo-lysosomal cholesterol accumulation and ER cholesterol depletion, thereby an activated SREBP2 signaling, in this condition (Ikonen, 2006). In the meanwhile, APP β-cleavage was enhanced in NPC1-depleted cells, as seen in decreased APPsα secretion and increased cellular C99 protein level (III, Figure 2F). Interestingly, no change in Aβ40 and Aβ42 levels in the medium was shown (III, Figure S1B). When APP was depleted together with NPC1, a further increase in cholesterol synthesis was observed (III, Figure 2E), indicating APP β-processing products imposed a negative regulatory role in SREBP2-regulated cholesterol synthesis.

3.4 APP ectodomains regulate cholesterol synthesis via the SREBP2 pathway

Due to the different regulatory effects of APP-M596V and APP-F615P on cholesterol synthesis, yet the same effect of their C-terminal fragments, we hypothesized that there could be a distinct biological function in APP N-terminal fragments, i.e. secreted APPsα and APPsβ ectodomains. Both APPsα and APPsβ were detectable in the conditioned medium of U251MG cells that had been depleted with endogenous APP and overexpressed with APP-M596V or APP-F615P constructs, respectively (III, Figure S2A). We used this conditioned medium as a donor medium to feed the APP-depleted acceptor cells (III, Figure 3A). In examining the cholesterol synthesis and SRE activity in acceptor cells, APP-M596V donor medium not only restored cholesterol synthesis, but also rescued SRE activity (III, Figures 3B and 3C); however, the APP-F615P donor medium did not affect cholesterol synthesis (III, Figure 3B). If APP-F615P donor medium acts as a negative regulator, the effect could be masked because of the significantly reduced basal cholesterol synthesis in the acceptor cells. Thus, we tested the APP-F615P donor medium on untreated U251MG acceptor cells (III, Figure 3D) and we observed attenuation in both cholesterol synthesis and SRE activity (III, Figures 3E and 3F).

To exclude the possibility that the donor medium contained molecules that secreted together with APPsα or APPsβ and acted in the signaling pathway of cholesterol synthesis, we used purified recombinant APPsα and APPsβ peptides to feed the acceptor cells as in the donor medium transfer experiments. We first measured the concentration of APPsα or APPsβ in the donor medium in previous experiments (III, Figure S2B). The 5-nM concentrations of both APPsα and APPsβ in the donor medium as detected by their specific antibodies were in the physiological range of APP ectodomain levels in the cerebrospinal fluid (Rosen et al., 2012). Recombinant APPsα and APPsβ peptides at this concentration were also effective in both cholesterol synthesis and SRE activity assays (III, Figure S2C). We next observed increased cholesterol synthesis and SRE activity in cholesterol-low cells cultured with APPsα peptide, but not with APPsβ (III, Figures S3A and 3G), and contrasting results in cholesterol-normal cells cultured with APPsβ peptide (III, Figures S3B and 3H). This result confirms that the two APP ectodomains function opposingly in cholesterol synthesis regulation via the SREBP2 pathway.

3.5 APP dose effect on cholesterol synthesis in liver cells

To further study the regulatory role of APP in SREBP2 signaling in cells specialized in sterol metabolism, we used a human hepatoblastoma cell line, HepG2, which is a commonly used cell model in lipid research. We started with depleting endogenous APP protein in HepG2 cells; however the knocking-down efficiency was not as high as in U251MG cells, partially because the endogenous APP level was higher in HepG2 cells. Therefore, we continued with overexpressing the wild-type APP in HepG2 cells by transient transfection and measured the subsequent cholesterol synthesis and LDLr SRE
activity by luciferase reporter assay. We observed enhanced cholesterol synthesis in APP-overexpressing HepG2 cells (III, Figure S3C). Increasing the APP protein level also doubled the LDLr SRE activity in the HepG2 cells, which indicates that APP has a dosage effect (III, Figure 4A). We then overexpressed APP-M596V or APP-F615P mutant constructs in the HepG2 cells to compare with the effects brought by wild-type APP overexpression. In accordance with our previous findings, overexpressing the APP-M596V increased the SRE luciferase activity. On the other hand, overexpressing the APP-F615P also led to an increase in SRE activity, but to a significantly lower extent compared with that of expressing wild-type APP or APP-M596V (III, Figure 4A), probably due to a high basal APP level. It suggests that, despite a dosage effect of APP, the difference between wild-type APP/APP-M596V as efficient positive regulators and APP-F615P as a less efficient regulator is significant. These results were confirmed in U251MG cells overexpressing these constructs (III, Figure 4A).

To characterize this regulation in HepG2 cells, we measured the LDLr protein level (III, Figure 4B) and Dil-LDL uptake (III, Figure 4C) in cells overexpressing either wild-type or mutant APP constructs. Not surprisingly, the LDLr protein level was increased and the Dil-LDL uptake was enhanced significantly in wild-type APP or APP-M596V-overexpressing cells, while the LDLr level was slightly but significantly decreased in APP-F615P-overexpressing cells.

We next fed HepG2 cells with donor medium from cells overexpressing wild-type or mutant APP constructs and examined the LDLr SRE activity, to test the role of APP ectodomains in cholesterol synthesis in HepG2 cells. In line with results from the LDLr uptake assay, wild-type APP or APP-M596V overexpression resulted in an increase in SRE activity, but APP-F615P overexpression did not (III, Figure 4D). Same observation was made using the recombinant APPs\(\alpha\) and APP\(s\beta\) peptides (III, Figure 4E). In an attempt to understand the underlying mechanism for the APP\(s\alpha\)-regulating SRE pathway, we analyzed GSK3\(\beta\) phosphorylation in acceptor cells. GSK3\(\beta\) is an important signaling molecule that receives signals converging via several pathways. The constitutively active GSK3\(\beta\) promotes proteasome degradation of mature SREBP2, and phosphorylation of GSK3\(\beta\) at S9 inhibits GSK3\(\beta\) activity (Du et al., 2006; Ying et al., 2012), thus enabling SREBP2 to be active in target gene transcription. By immunoblotting using a phosho-specific antibody, we found an increase in GSK3\(\beta\) phosphorylation at S9 in acceptor HepG2 cells treated with APP-M596V donor medium for 1 h (Figure 7). This points out a signaling response in cells upon APP\(s\alpha\) ectodomain stimulation, rendering a rapid response in cholesterol balance regulation. Future study of the signaling pathway in further detail would be beneficial.

*Figure 7. GSK3\(\beta\) phosphorylation at S9 in HepG2 cells upon 1 h incubation with or without APP-M596V donor medium (n=6).*
3.6 SREBP2 targets in familial AD patients with APP duplication

Having observed the dosage effect of APP on SREBP2 signaling in HepG2 cells, we then aimed at this regulatory change under naturally occurring conditions at high APP dosages. We obtained human primary fibroblasts from APP<sup>Dp</sup> patients as a model for this study.

These APP<sup>Dp</sup> patients were found in four generations of a Finnish family, exhibiting clinical features, including progressive cognitive decline, characteristic of AD. The patients were characterized as having an APP locus duplication rather than APP gene point mutation (Rovelet-Lecrux et al., 2007). Therefore, it suggests that APP has a dosage effect on the AD pathology manifested in APP<sup>Dp</sup> patients.

To carry out this study, we chose APP<sup>Dp</sup> primary fibroblasts from two patients who had not been treated with cholesterol-lowering statin, and two commercial human primary fibroblasts (GM323 and AG) as controls. We first tested if we could reproduce APP depletion-induced LDLr protein level reduction as observed in U251MG cells. About 60% of the endogenous APP protein in APP<sup>Dp</sup> fibroblasts was depleted by siRNA transient transfection, while the LDLr protein level was decreased by ~30% (III, Figure 5A). We then compared the endogenous APP and LDLr protein levels in control and APP<sup>Dp</sup> fibroblasts. Although the APP level in one control fibroblast cell line was not significantly lower than in both APP<sup>Dp</sup> fibroblast cell lines, the LDLr levels were strikingly lower in APP<sup>Dp</sup> fibroblasts than in the controls (III, Figure 5B). Considering our previous findings that β-processing of APP downregulates the SREBP2 pathway, the lowered LDLr protein level in APP<sup>Dp</sup> fibroblasts indicates that endogenous APP may experience enhanced β-cleavage.

In a recently published article (Ikonen, 2008) examining the reprogramming of APP<sup>Dp</sup> primary fibroblasts into induced pluripotent stem cells (iPSCs), iPSC-derived neurons significantly increased the numbers of Rab5-positive large early endosomes (>1 μm<sup>3</sup> in volume) relative to controls. Endosome dysfunction and enlarged size were found in cells from a Down's syndrome patient with an extra copy of the APP gene, and this abnormality in endosome morphology and function could be corrected by inhibiting BACE1 (Jiang et al., 2010). We observed a similar endosome phenotype in APP<sup>Dp</sup> fibroblasts. By staining control and patient fibroblasts with anti-EEA1 antibody, we found that in APP<sup>Dp</sup> fibroblasts, EEA1-positive large early endosomes (>1 μm<sup>2</sup>) comprised ~18% of the total endosomes, but only ~8% in control fibroblasts (III, Figure 5C). This suggests β-processing of APP may be favored in APP<sup>Dp</sup> fibroblasts. To test this hypothesis, we treated APP<sup>Dp</sup> fibroblasts with BACE1 inhibitor or siRNA oligos and measured the cellular LDLr protein level by Western blotting. An increase in the LDLr level was seen after either treatment (III, Figure 5D), indicating that APP β-processing caused suppression of the LDLr protein level in APP<sup>Dp</sup> fibroblasts, at least partially. In accordance with this, we observed increased Dil-LDL uptake in BACE inhibitor-treated APP<sup>Dp</sup> fibroblasts (III, Figure 5E).

To understand the correlation between sterol levels and APP duplication, we obtained serum samples collected from two APP<sup>Dp</sup> patients who were the donors of the primary fibroblasts. We analyzed the serum sterol levels by gas-liquid chromatography (III, Table 1). The cholesterol levels in the sera from these two patients were in the range of those from healthy individuals. However, the ratios of other minor circulating sterols to cholesterol are more specific indicators in studying cholesterol metabolism (Miettinen et al., 2011). To assess cholesterol absorption, plant sterol (cholesterol, campesterol, and sitosterol) to cholesterol ratios were determined and they were all in the range of
reference values, suggesting that cholesterol absorption was not impaired in APP\textsuperscript{Dp} patients. For cholesterol \textit{de novo} synthesis, squalene and cholesterol precursor (lathosterol and desmosterol) to cholesterol ratios were measured. In both APP\textsuperscript{Dp} patient serum samples, the squalene:cholesterol and lathosterol:cholesterol ratios fell out of the reference value range, indicating that cholesterol synthesis was weakened. Interestingly, the desmosterol:cholesterol ratio was normal in both samples. This is in line with the alternative regulation of desmosterol via LXR-regulated DHCR24 activity, apart from the conventional SREBP2-mediated control over the squalene and lathosterol levels.

Together, these results suggest that in fibroblasts where sterols are not actively synthesized, APP β-processing also plays a role in regulating the LDLr level and LDL uptake, presumably via the SREBP2 pathway.

3.7 APP knockdown in primary astrocytes

Since ABCA1 and ApoE are the two proteins in charge of cholesterol efflux, we aimed at determining their cellular levels upon APP\textsubscript{α} addition. Since the ApoE protein level is too low to observe in both U251MG and CCF astrocytoma cell lines (unpublished data), we used primary astrocytes from embryonic mouse brain for this purpose. We did not observe a significant change in the ABCA1 level in the astrocytes with 20-h APP\textsubscript{α} incubation, leaving open the possibility of responses from other ABC family proteins. Indeed, a previous report showed that the cholesterol efflux from cerebellar astrocytes is different from that in peripheral cells, and it does not correlate with ABCA1 expression (Karten et al., 2006). We likewise did not observe a change in the cellular ApoE level; however, the ApoE level in the medium was significantly increased. This means that either astrocytes generate more ApoE and secrete it to facilitate cholesterol efflux, or more ApoE is stabilized in the medium (Figure 8).

\textit{Figure 8. ApoE levels in primary astrocytes incubated with APP\textsubscript{α}. Western blotting and band intensity quantification for (A) ApoE in mouse primary astrocytes and (B) conditioned astrocyte medium. The cells were incubated with 5 nM APP\textsubscript{α} for 20 h in serum-free medium before collecting for analysis.}

3.8 Proposed model for the role of APP in cholesterol balance in the CNS

Based on the literature and our findings, we proposed a model for cholesterol balance regulation by APP in the neuron-astrocyte context (Figure 9). Astrocytes, as sterol
providers for neuronal cells, synthesize cholesterol and secret it via ApoE-containing lipoproteins particles. Neurons, on the other hand, are more considered as sources of APP and the recipients of exogenous cholesterol generated by astrocytes. When the neuronal cholesterol content is low, APP undergoes processing via the non-amyloidogenic pathway. APP is cleaved in the PM by α-secretase and the APPsα generated is released from the cell. APPsα stimulates SREBP2 activity in the surrounding astrocyte(s), presumably via a signaling cascade. This helps astrocytes to increase their cholesterol synthesis to deliver increasing amounts of sterol to neurons via ApoE lipoprotein particles. Upon γ-cleavage, the intracellularly released AICD in turn activates SREBP2-dependent gene transcription, leading to increased neuronal cholesterol production. When the neuronal cholesterol content increases over a threshold - with endosomal cholesterol serving as a likely barometer - APP becomes increasingly processed via the amyloidogenic pathway within the endosomal compartments. APPsβ and Aβ, the cleavage products of APP by β- and γ-secretases, are secreted from neurons and suppress SREBP2-dependent transcription in neighboring astrocytes. In this way, APP downregulates astrocyte sterol production and reduces neuronal sterol delivery from astrocytes. In cholesterol-enriched neurons, intracellular APP signaling also reduces cholesterol. This involves AICD-mediated negative regulation of SREBP2 signaling, presumably in cooperation with a different set of proteins than in the case of AICD generated by the α-secretase pathway.

Figure 9. Model of APP-regulated cholesterol metabolism.
Conclusions and Future Prospects

In this study, we investigated how DHEA-FAE-LDL is processed in the cell by comparing it to CE processing. DHEA-FAE-LDL uptake is antagonized by excess LDL, suggesting an LDLr or LDLr-related uptake of DHEA-FAE-LDL. We also identified the metabolites of DHEA-FAE after hydrolysis. While the cellular DHEA-FAE level decreases, suggesting it is being processed, free DHEA and two metabolites, 5α-androstanediol and androstenedione, are secreted into the medium. To understand DHEA-FAE hydrolysis, we assessed the LAL activity in hydrolyzing DHEA-FAE, since LAL is the enzyme that hydrolyzes LDL-borne CE. LAL exhibits modest activity in hydrolyzing DHEA-FAE compared with CE, implying that there are other players involved in this process. Hormone-sensitive lipase could be one of these players in DHEA-FAE hydrolysis, which may require multiple lipase activities, and future study on this would be beneficial.

Desmosterol accumulates in the developing brain of several species. We combined the literature and our own findings and provided evidence for the cause and consequences of desmosterol accumulation during the development of the mammalian brain. First, desmosterol accumulation could be caused by posttranscriptional repression of DHCR24 by progesterone. Second, desmosterol accumulation may promote sterol secretion from astrocytes in the brain by activating LXR signaling, thereby increasing the ABCA1 protein level. Third, desmosterol accumulation could serve to enhance the levels of active brain sterols by reducing the formation of inert sterol esters and secretory 24S-hydroxysterols. Together, these processes may assist in the rapid accumulation and distribution of sterols that are required in the developing brain.

Cholesterol influences APP processing, and in turn the APP proteolytic products Aβ and AICD are known to regulate cholesterol balance. This study investigated the direct role of APP in cholesterol homeostasis by acute manipulation of APP and proteolytic fragment levels. We showed that APP regulates cholesterol synthesis and uptake via the SREBP2 pathway, and unraveled the differential regulation via α- vs. β-processing of APP. While no difference was seen in APP C-terminal fragments produced via α- vs. β-cleavage, we identified N-terminal ectodomains as the new regulators of cholesterol synthesis. APPα enhances and APPβ inhibits cholesterol synthesis via the SREBP2 pathway. We also provided evidence that APPα promoted mature SREBP2 stabilization by GSK3β inhibitory phosphorylation. To gain further insight into the mechanism of APPsα-inactivating GSK3β, the upstream kinase activities in the GSK3β signaling pathways need to be analyzed. Additionally, there may be other non-GSK3β signaling pathways involved in parallel. The cellular receptor for APP ectodomains and how they trigger the signaling pathways also need further elucidation. The role of APP processing in regulating cholesterol homeostasis is ubiquitous, based on our observations in astrocytic, hepatocytic, and fibroblastic cells. In APPPDP FAD patient serum samples, we observed a decrease in the ratio of circulating cholesterol precursors to cholesterol, indicating impaired cholesterol synthesis. We proposed the possibility that the ratios of circulating cholesterol precursors to cholesterol may reflect APP amyloidogenesis and may serve as an early biomarker for AD.
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