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Cholesterol Disturbances in Inherited Neurodegenerative Diseases

Studies on *Npc1*, *Ppt1* and *Ctsd* deficient mice

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ACADEMIC DISSERTATION

To be publicly discussed with the permission of the Medical Faculty of the University of Helsinki, in the 5th Lecture Hall of the Helsinki University Main Building, on December 18th, 2009 at 12 noon.

Helsinki 2009

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Science is a way of thinking much more than it is a body of knowledge.

Carl Sagan

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Abstract

The central nervous system (CNS) is the most cholesterol-rich organ in the body. Cholesterol is essential to CNS functions such as synaptogenesis and formation of myelin. Significant differences exist in cholesterol metabolism between the CNS and the peripheral organs. However, the regulation of cholesterol metabolism in the CNS is poorly understood compared to our knowledge of the regulation of cholesterol homeostasis in organs reached by cholesterol-carrying lipoprotein particles in the circulation.

Defects in CNS cholesterol homeostasis have been linked to a variety of neurodegenerative diseases, including common diseases with complex pathogenetic mechanisms such as Alzheimer's disease. In spite of intense effort, the mechanisms which link disturbed cholesterol homeostasis to these diseases remain elusive. We used three inherited recessive neurodegenerative disorders as models in the studies included in this thesis: Niemann-Pick type C (NPC), infantile neuronal ceroid lipofuscinosis and cathepsin D deficiency. Of these three, NPC and cathepsin D deficiency have previously been linked to disturbed cholesterol metabolism. Elucidating the mechanisms with which disturbances of cholesterol homeostasis link to neurodegeneration in recessive inherited disorders with known genetic lesions should shed light on how cholesterol is handled in the healthy CNS and help to understand how these and more complex diseases develop.

In the first study we analyzed the synthesis of sterols and the assembly and secretion of lipoprotein particles in *Npc1* deficient primary astrocytes. We found that both wild type and *Npc1* deficient astrocytes retain significant amounts of desmosterol and other cholesterol precursor sterols as membrane constituents. No difference was observed in the synthesis of sterols and the secretion of newly synthesized sterols between *Npc1* wild type, heterozygote or knockout astrocytes. We found that the incorporation of newly synthesized sterols into secreted lipoprotein particles was not inhibited by *Npc1* mutation, and the lipoprotein particles were similar to those excreted by wild type astrocytes in shape and size. The bulk of cholesterol was found to be secreted independently of secreted NPC2. These observations demonstrate the ability of *Npc1* deficient astrocytes to handle *de novo* sterols, and highlight the unique sterol composition in the developing brain.

Infantile neuronal ceroid lipofuscinosis is caused by the deficiency of a functional Ppt1 enzyme in the cells. In the second study, global gene expression studies of approximately 14000 mouse genes showed significant changes in the expression of 135 genes in *Ppt1* deficient neurons compared to wild type. Several genes encoding for enzymes of the mevalonate pathway of cholesterol biosynthesis showed increased expression. As predicted by the expression data, sterol biosynthesis was found to be upregulated in the knockout neurons. These data link *Ppt1* deficiency to disturbed cholesterol metabolism in CNS neurons.

In the third study we investigated the effect of cathepsin D deficiency on the structure of myelin and lipid homeostasis in the brain. Our proteomics data, immunohistochemistry and western blotting data showed altered levels of the myelin protein components myelin basic protein, proteolipid protein and 2', 3'-cyclic nucleotide 3' phosphodiesterase in the brains of cathepsin D deficient mice. Electron microscopy revealed altered myelin structure in cathepsin D deficient brains. Additionally, plasmalogen-derived alkenyl

chains and 20- and 24-carbon saturated and monounsaturated fatty acids typical for glycosphingolipids were found to be significantly reduced, but polyunsaturated species were significantly increased in the knockout brains, pointing to a decrease in white matter. The levels of ApoE and ABCA1 proteins linked to cholesterol efflux in the CNS were found to be altered in the brains of cathepsin D deficient mice, along with an accumulation of cholesteryl esters and a decrease in triglycerols. Together these data demonstrate altered myelin architecture in cathepsin D deficient mice and link cathepsin D deficiency to aberrant cholesterol metabolism and trafficking.

Basic research into rare monogenic diseases sheds light on the underlying biological processes which are perturbed in these conditions and contributes to our understanding of the physiological function of healthy cells. Eventually, understanding gained from the study of disease models may contribute towards establishing treatment for these disorders and further our understanding of the pathogenesis of other, more complex and common diseases.

Contents

List of original publications

This thesis is based on the following original publications, which are referred to in the text by their corresponding roman numerals.

I) Mutka AL, Lusa S, Linder MD, Jokitalo E, Kopra O, Jauhiainen M, Ikonen E. (2004) Secretion of sterols and the NPC2 protein from primary astrocytes. Journal of Biological Chemistry. 279(47):48654-62.

II) Ahtiainen L, Kolikova J, Mutka AL, Luiro K, Gentile M, Ikonen E, Khiroug L, Jalanko A, Kopra O. (2007) Palmitoyl protein thioesterase 1 (Ppt1) deficient mouse neurons show alterations in cholesterol metabolism and calcium homeostasis prior to synaptic dysfunction. Neurobiology of Disease. 28(1):52-64.*

III) Mutka AL**, Haapanen A**, Käkelä R, Lindfors M, Inkinen T, Hermansson M, Rokka A, Jauhiainen M, Ikonen E, and Tyynelä J. (2009) Cathepsin D deficiency is associated with extreme demyelination and accumulation of cholesteryl esters in the brain. Journal of Neurochemistry. In press.

* Published previously as a part of Laura Ahtiainen's dissertation Unravelling Molecular and Cellular Disease Mechanisms in Infantile Neuronal Ceroid Lipofuscinosis (INCL).

** The authors contributed equally to the study

Abbreviations

ABC	ATP-binding cassette
ACAT	Acyl coenzyme A:cholesterol acyltransferase
AD	Alzheimer's disease
Apo	Apolipoprotein
ApoER2	Apolipoprotein E receptor 2
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
cAMP	Cyclic adenosine monophosphate
CatD	Cathepsin D
CNS	Central nervous system
CoA	Coenzyme A
CSF	Cerebrospinal fluid
DCC	Deleted in colon cancer
DHCR24	3-β-hydroxysterol-Δ(24)-reductase
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
FXR	Farnesoid X receptor
GFAP	Glial fibrillary acidic protein
HC	Hippocampus
HDL	High density lipoprotein
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HMG-CoAR	HMG-CoA reductase
HPLC	High performance liquid chromatography
HP-TLC	High performance thin layer chromatography
HZ	Heterozygote
INCL	Infantile neuronal ceroid lipofuscinosis
Insig	Insulin induced gene
kDa	Kilodalton
KO	Knockout
LBPA	Lysobisphosphatidic acid
LCAT	Lechithin-cholesterol acyl transferase
LDL	Low density lipoprotein
LDLR	LDL receptor
LR11/SORLA	Sortilin-related receptor LDLR class A
LRP	LDL receptor-like protein
LXR	Liver X receptor
MBP	Myelin basic protein
NADPH	Nicotinamide adenine dinucleotide phosphate
NCL	Neuronal ceroid lipofuscinosis
NgBR	Nogo-B receptor

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NPC	Niemann-Pick disease type C
ORF	Open reading frame
OSBP	Oxysterol binding protein
PBS	Phosphate buffered saline
PM	Plasma membrane
Ppt1	Palmitoyl-protein thioesterase 1
RAP	Receptor associated protein
RXR	Retinoid X receptor
SCAP	SREBP cleavage activating protein
Seladin	Selective Alzheimer's disease indicator
SFV	Semliki Forest virus
SNARE	Soluble N-ethylmaleimide-sensitive attachment protein receptor
SR-B	Class B scavanger receptor
SRE	Sterol regulatory element
SREBP	Sterol regulatory element binding protein
SSD	Sterol-sensing domain
SV	Synaptic vesicle
VAMP	Vesicle associated membrane protein
VLDLR	Very low density lipoprotein receptor
WT	Wild type

Introduction

1. Cholesterol in the cell

Cholesterol is a ubiquitous and integral constituent of mammalian cell membranes and essential to normal cell function and survival. In addition to being a membrane component, cholesterol is utilized as a precursor for other biomolecules, such as vitamin D, steroid hormones and bile acids. It also participates in signaling events by modifying proteins, such as the morphogenetic factor Hedgehog (Jeong and McMahon, 2002). Disturbances in cellular cholesterol metabolism have been implicated in a variety of pathological conditions, including common diseases such as atherosclerosis (Berenson et al., 1998), and rare disorders such as familial hypercholesterolaemia (Mabuchi et al., 1989), desmosterolosis (FitzPatrick et al., 1998), and many others. In spite of intense research effort over decades, many questions remain as to how cells handle cholesterol. This is particularly true for cholesterol metabolism in the central nervous system (CNS). In this thesis I have focused on cholesterol metabolism in inherited neurodegenerative diseases.

1.1. The lipid bilayer

Most cellular membranes are composed of lipid bilayers. Its basic components are amphiphatic lipid molecules, which spontaneously form micelles and bilayers when exposed to an aqueous environment. The polar, hydrophilic head groups of the molecules face the aqueous environment and the hydrophobic tails face the center of the bilayer as this is the most energetically favorable configuration. In cells, the bulk of cholesterol is in membranes. Lipid membranes line all cell organelles, compartmentalize cellular functions and separate the cell from the environment. Lipids constitute more than half of cell membrane mass, with the remainder being mostly protein.

The main groups of lipids in the membranes are glycerolipids, sphingolipids and cholesterol. Glycerolipids and sphingolipids share structural similarities. They both have a hydrophilic head group, an interface region and two long hydrocarbon chains which form the hydrophobic domain of the molecules. The glycerolipids contain a glycerol backbone, with two long-chain fatty acids linked through ester bonds to adjacent carbon atoms. The third carbon is linked to a phosphate group, which in turn is linked to one of several different head groups. By combining different fatty acids and head groups cells can make several types of glycerolipids. Sphingolipids are further classified as phosphosphingolipids or glycosphingolipids according to the type of headgroup attached to their C1 carbon. The phosphosphingolipid species in animal cells is sphingomyelin, which is derived from sphingosine and has phosphocholine as the head group (Image A).

Cholesterol is a 27-carbon tetracyclic molecule (Image A). Its properties are characterized by its small size, hydrophobicity and rigidity. In cell membranes, cholesterol occupies the spaces between the hydrocarbon chains of phospholipids. In doing so,

cholesterol condenses the packing of the membrane components and regulates membrane fluidity and permeability. In the membrane, cholesterol is oriented by the 3β-OH group, which gives the otherwise hydrophobic molecule a somewhat amphipatic character. The hydroxyl group can also mediate the hydrogen bonding between cholesterol and water and other lipid components in membranes (Ohvo-Rekila et al., 2002).
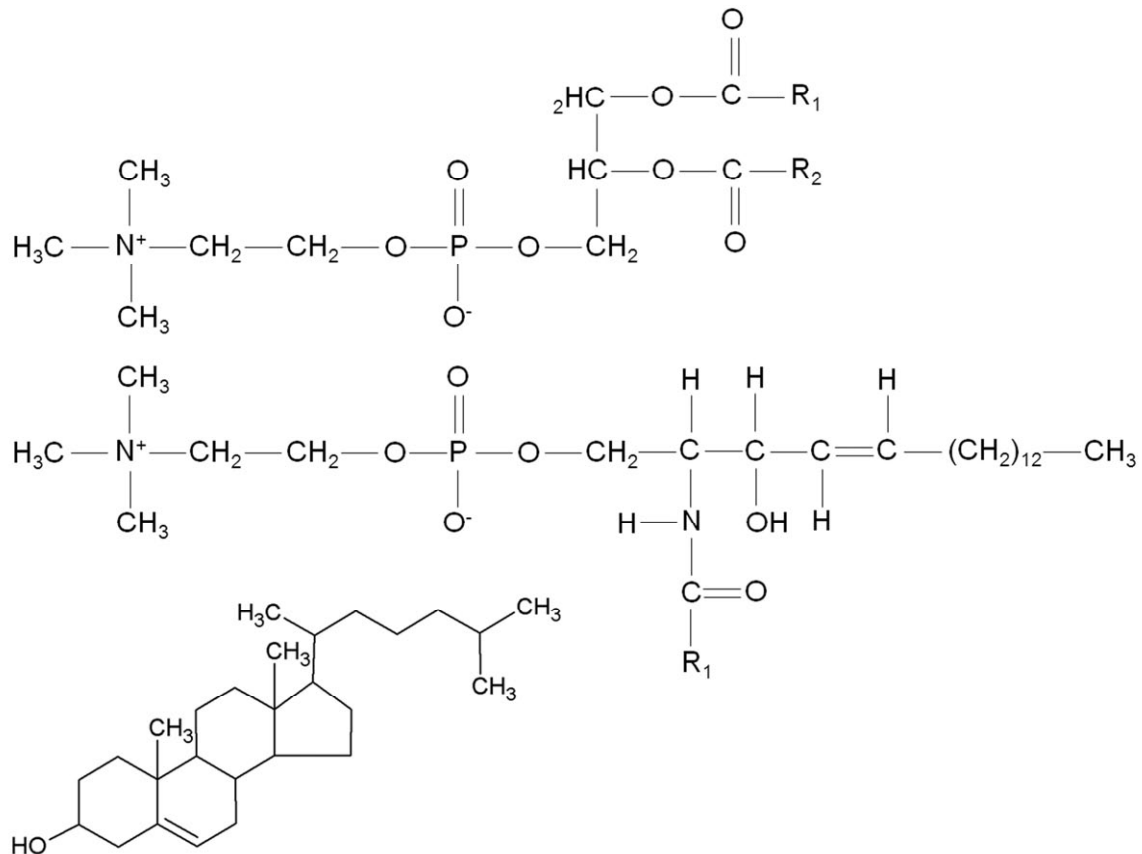
Image A: Structures of phosphatidylcholine (top), sphingomyelin (middle) and cholesterol (bottom). R_1 and R_2 represent the hydrocarbon chains of fatty acids.

Lipids are not symmetrically arranged on the membranes. Glycolipids are present in the external leaflet of the plasma membrane. The external leaflet is also enriched with sphingomyelin and phosphatidylcholine (lecithin), whereas phosphatidylethanolamine and phosphatidylserine are mostly found in the inner leaflet (Voelker, 2002) (Image B). Cholesterol too is asymmetrically distributed. The transbilayer distribution of cholesterol has been a point of controversy: several studies suggest that the majority of cholesterol is found on the external leaflet of the plasma membrane (Wood et al., 1999), but some studies suggest that up to 70% of plasma membrane cholesterol resides in the cytoplasmic leaflet (Mondal et al., 2009). The majority of cholesterol is thought to reside in the plasma membrane (Lange, 1991). There is evidence for a cholesterol gradient in the membranes of the Golgi complex with an increase in the cis-trans direction (Bretscher and Munro, 1993), and there are differences in the cholesterol content of different organelles of the

endosomal pathway (Lange, 1991; Hao et al., 2002; Mobius et al., 2003). The endoplasmic reticulum (ER) is thought to be relatively sterol-poor and the changes in its cholesterol content represent a key variable in governing cell cholesterol homeostasis (DeBose-Boyd et al., 1999). The inner leaflets of mitochondria are also sterol-poor. Consequently, the transfer of cholesterol to P450 side-chain cleavage enzyme, which is the the first enzyme of the steroid hormone synthesis pathway and resides on the inner leaflet of the mitochondrial membrane, is the rate-limiting step in steroidogenesis (Lin et al., 1995). In polarized cells, there are also differences between the cholesterol content of plasma membrane domains (Danielsen and Hansen, 2006). The mechanisms which keep up the subcellular distribution of cholesterol may be linked to the distribution of sphingolipids in the cell, as cholesterol preferentially interacts with sphingomyelin in the membranes (Ohvo-Rekila et al., 2002), or be due to transport mechanisms within the cell (Ikonen, 2008).
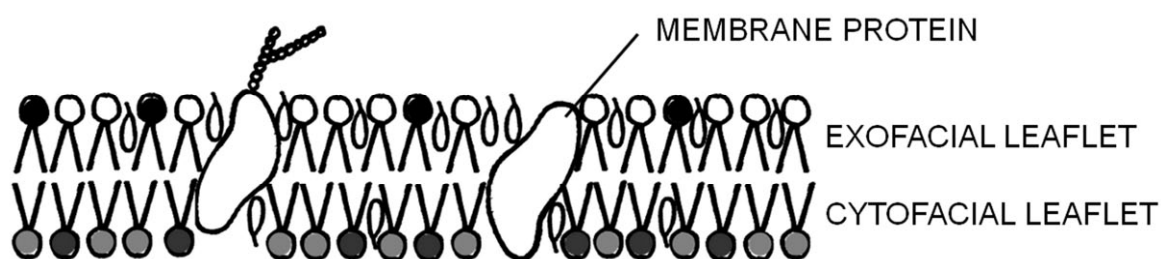

Image B: A schematic illustration of the lipid bilayer characteristic to the plasma membrane. Cholesterol (small ovals) is found on both leaflets. The external leaflet is enriched in sphingolipids (white) and phosphatidylcholine (black), whereas the cytofacial leaflet is enriched in phosphatidylserine (gray) and phosphatidylethanolamine (dark gray).

In the fluid-mosaic model of biomembrane structure, it was considered that bilayers are in a fluid state and lipids and proteins are homogenously distributed in the membrane (Singer and Nicolson, 1972). Current view holds that biological membranes are asymmetrical and contain microdomains with distinct lipid and protein compositions (Simons and Ikonen, 1997). Sphingolipids typically contain longer, more saturated fatty acids as compared to membrane glycerophospholipids. Cholesterol preferentially interacts with more saturated hydrocarbon chains in membranes, and as a result cholesterol and sphingolipids spontaneously cluster together in dynamic lateral assemblies. These liquid ordered (L_o) domains are thought to be fluid yet more tightly packed than the surrounding membrane, which exists in a liquid disordered (L_d) state (Brown and London, 2000). L_d membranes are more ready to dissolve in mild detergents than L_o domains because of their looser packing. The laterally organized moving platforms rich in cholesterol and sphingolipid have been termed lipid rafts (Simons and Ikonen, 1997). In a biochemical and cell biological context, lipid rafts have been defined by their low density and resistance to solubility in cold nonionic detergents, such as Triton X-100 (Brown and Rose, 1992; Simons and Ikonen, 1997). The membranes which can be isolated with this technique are called detergent resistant membranes (DRM).

Some proteins preferentially partition into rafts (Bickel et al., 1997) and the function or processing of some proteins may depend on raft association (Ehehalt et al., 2003; Cheng et al., 2007). Proteins themselves may play an active role in the formation of functional membrane microdomains (Okamoto et al., 1998). Rafts have been implicated in a variety of cellular functions, such as signal transduction, cell adhesion and vesicle trafficking (Simons and Ikonen, 1997). However, due to technical difficulties in the isolation and study of rafts, there has been considerable controversy as to their nature (Munro, 2003). In recent years, advances in techniques such as live-cell microscopy have deepened our knowledge of the nature and function of rafts. A current view holds that rafts are small (10-200nm), heterogenous and highly dynamic domains enriched in sterols and sphingolipids which compartmentalize cellular processes, and can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions (Pike, 2006).

1.2. How cells acquire cholesterol

1.2.1. Uptake of lipoproteins

Cells acquire cholesterol by taking up cholesterol-containing lipoproteins via receptor-mediated endocytosis (Brown and Goldstein, 1986) (Image C). In cells outside of the CNS, ApoB-100-containing low-density lipoprotein particles (LDL), which carry cholesterol mostly as cholesteryl esters (CE), are identified by LDL receptors (LDLR). These receptors are associated with clathrin-coated pits on the plasma membrane. The LDL particles are internalized and enter the endosomal system. The receptor dissociates from the particle in early endosomes and recycles back to the plasma membrane (Maxfield and McGraw, 2004). In the acidic environment of the late endosome/lysosome compartment, acid lipase catalyzes the hydrolysis of the lipoprotein-derived cholesteryl esters to free cholesterol and fatty acids (Goldstein et al., 1975). After this stage, the fate of the lipoprotein-derived cholesterol is less well understood. Movement of cholesterol between subcellular compartments and membranes is essential for cholesterol to reach the metabolically active pool and be stored in lipid droplets, mostly as cholesteryl esters. Cholesterol can move from the endosomes to the plasma membrane and it can be transported to the ER for esterification (Lange et al., 1997). Transport of LDL-derived cholesterol from the late endosomes/lysosomes may involve the trans-golgi network (Urano et al., 2008). If the limiting membranes of endosomes become saturated with cholesterol, cholesteryl ester hydrolysis stalls and cholesterol is re-esterified (Wang et al., 2005). Additionally, if either of the two Niemann-Pick type C (NPC) proteins, NPC1 and NPC2, is not functional, free cholesterol accumulates in the late endosomes (Vance, 2006). The NPC proteins are introduced in more detail later in this review. Recently, the sterol-responsive nuclear liver X receptors (LXR) were shown to inhibit the LDLR pathway through transcriptional regulation of Inducible degrader of the LDLR (Idol), which triggers ubiquitinylation of LDLR (Zelcer et al., 2009).
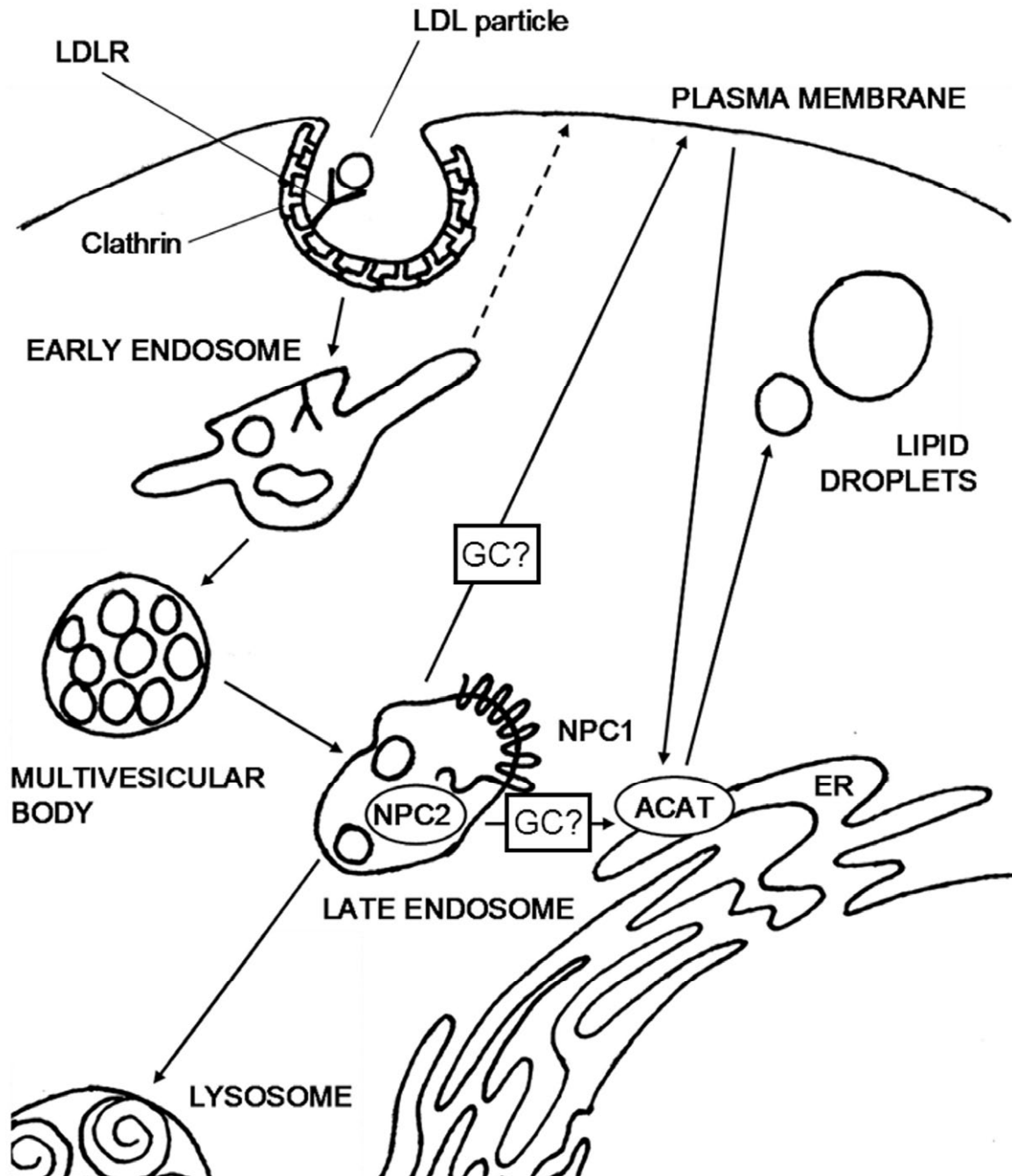
Image C: Uptake of LDL-derived cholesterol by receptor-mediated endocytosis and the fate of LDL-derived cholesterol. LDL particles enter the endosomal system bound to LDLR via clathrin-coated pits. LDL dissociates from LDLR in early endosomes, and the receptors are recycled to the plasma membrane (dashed line) or degraded. Free cholesterol is hydrolysed from CE in the late endosomes/lysosomes by acid lipase. Cholesterol then moves from NPC2 and NPC1-containing late endosomes to the plasma membrane or to the ER for esterification by acyl coenzyme A:cholesterol acyltransferase (ACAT). Transport of cholesterol from the endosomal system may involve the Golgi complex (GC). Cholesteryl esters are stored in lipid droplets.

1.2.2. The biosynthesis of sterols

All nucleated mammalian cells are capable of synthesizing cholesterol. Cholesterol is synthesized from acetyl-coenzyme A (acetyl-CoA) via the mevalonate pathway. In addition to sterols and sterol derivatives, the pathway also produces isoprenoids and participates in the generation of products such as heme A, ubiquinone and farnesylated and geranylgeranylated proteins (Edwards and Ericsson, 1999). The rate-limiting step of the mevalonate pathway is the conversion of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonate (Berg et al., 2002). The enzyme responsible for this conversion is the HMG-CoA reductase (HMG-CoAR). HMG-CoAR is the target for the widely used statin class of hypercholesterolaemia drugs, which inhibit its activity (Endo, 1992).

The cholesterol synthesis pathway is complex and it takes about 30 enzymatic steps for the first precursor, acetyl-CoA, to be converted into the end-product cholesterol. The majority of this complexity presents quite late in the pathway. It takes 19 steps to convert lanosterol, the first intermediate with a sterol backbone, to cholesterol (Gaylor, 2002). After lanosterol, the pathway splits into two alternative routes: the Bloch pathway via desmosterol and the Kandutch-Russell pathway via lathosterol and 7-dehydrocholesterol (Image D). If at any point in the Bloch pathway an intermediate undergoes a Δ24-reduction, it is shifted to the Kandutch-Russell pathway. Knocking out Δ24-reductase activity will result in the accumulation of the penultimate cholesterol precursor, desmosterol (Wechsler et al., 2003) (Image D). However, 3-β-hydoxysterol-Δ(24)-reductase (DHCR24), the enzyme responsible for the Δ24-reduction, binds preferentially to the penultimate sterol intermediates of the pathway so even if the Δ24-reduction of earlier intermediates is possible, it does not appear to be favored (Pedretti et al., 2008).

Most steps of cholesterol synthesis take place in the ER, but some have been localized to peroxisomes (Keller et al., 1986). This compartmentalization does not appear to be essential as cholesterol biosynthesis is not perturbed in mice lacking functional peroxisomes (Hogenboom et al., 2002). Most cell cholesterol resides in the plasma membrane (Liscum and Munn, 1999). Therefore, transport mechanisms to move newly synthesized cholesterol from the ER and the late endosomes/lysosomes to the plasma membrane must exist. Newly synthesized cholesterol reaches the plasma membrane rapidly via mechanisms which are mostly independent of the Golgi complex (Heino et al., 2000). However, an intact cytoskeleton is required because if the integrity of the actin cytoskeleton is pharmacologically compromised, the release of newly synthesized cholesterol is inhibited (Lusa et al., 2003). Once newly synthesized cholesterol reaches the plasma membrane, it can be shuttled between the plasma membrane and recycling endosomes (Hao et al., 2002) or transported to the ER for esterification (Lange et al., 1997).
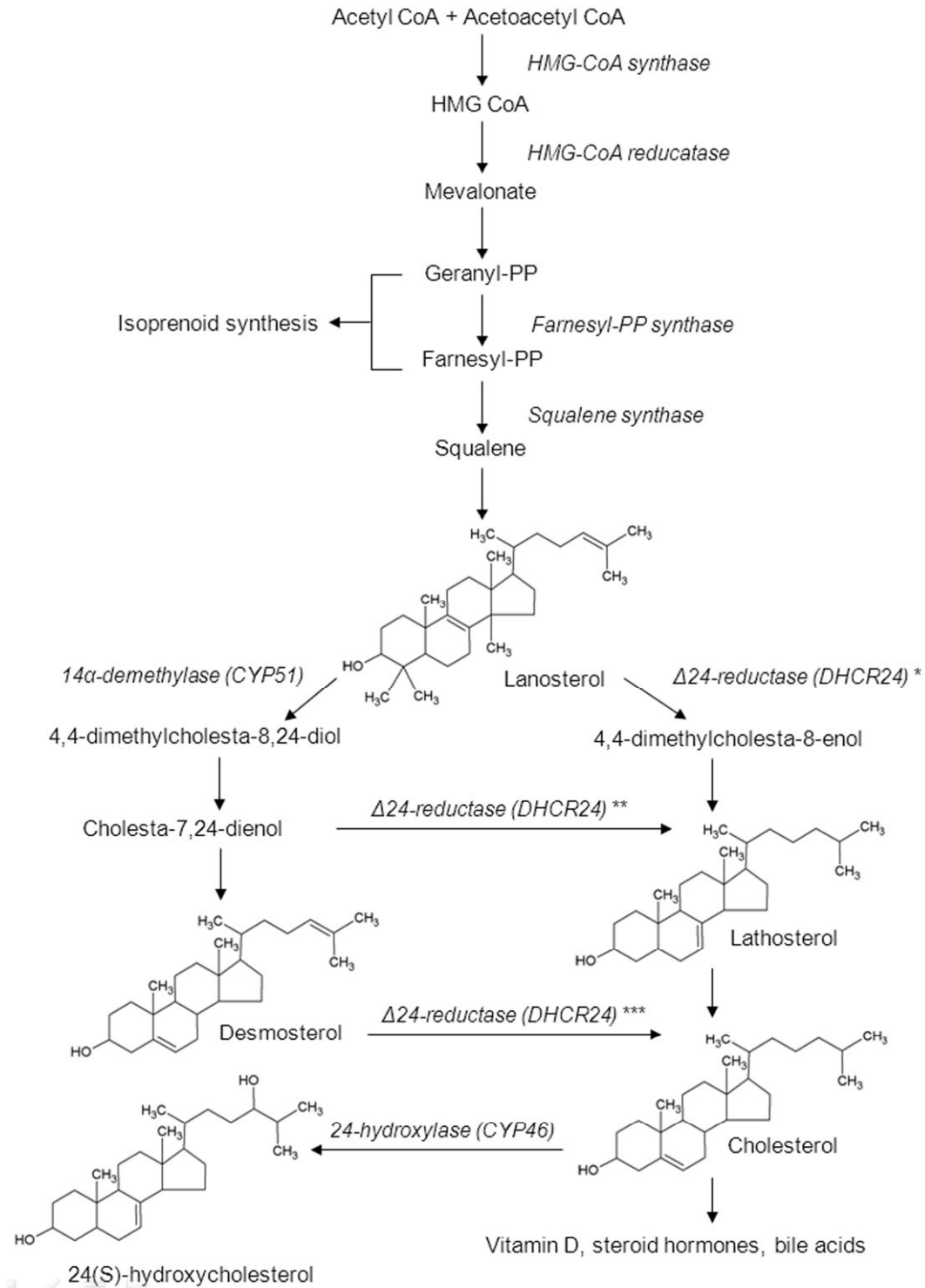
Image D: A simplified illustration of cholesterol biosynthesis. Where no enzyme is specified, the conversion takes two or more enzymatic steps. * CYP51 activity also required in this conversion (14-demethylation). ** Preferred reaction according to (Bae and Paik, 1997). * Preferred reaction according to (Pedretti et al., 2008).**

The biosynthesis of cholesterol is rigorously controlled. Transcriptional control is mediated by regulating the transport of sterol regulatory element binding proteins (SREBP) from the ER to the Golgi complex. These transcription factors then undergo proteolytic cleavage, move to the nucleus and activate the transcription of several genes involved in cholesterol synthesis and uptake (Brown and Goldstein, 1999). There are three ubiquitous SREBP proteins currently identified: SREPB-1a and -1c are produced from a single gene through the use of alternate promoters and the third, SREBP-2, is produced from a separate gene (Hua et al., 1995). A sterol-insensitive, germ-cell enriched isoform of SREBP-2 has also been identified and termed SREBP-2gc (Wang et al., 2002). The sterol-sensing SREBP cleavage activating protein (SCAP) binds SREBPs and regulates their function by controlling their ER-Golgi transport in a sterol-sensitive manner (Brown and Goldstein, 1999).

When ER sterol levels are low, SCAP escorts SREBP to the Golgi complex to undergo sequential cleavage by two proteases (DeBose-Boyd et al., 1999). This releases the active NH2-terminal fragment, which then enters the nucleus and binds to sterol regulatory element (SRE), the promoter sequence found in the SRE target genes (Image E). Reciprocally, when sterols accumulate in the ER, SCAP changes conformation in a way that favors binding to the integral membrane proteins Insig-1 and Insig-2, which in turn results in the retention of the SCAP/SREBP complex in the ER (Brown et al., 2002; Yang et al., 2002). Structural analysis suggests that once SCAP is bound to Insig, the hexapeptide sorting signal in SCAP is rendered inaccessible to COPII proteins, which serve as coat proteins in ER-Golgi transport vesicles (Sun et al., 2007). The presence of increased amounts of sterols or mevalonate forms a feed-back loop and leads to the rapid degradation of existing HMG-CoAR molecules via the ubiquitin-proteasome pathway (Ravid et al., 2000).

The different isoforms of SREBPs regulate in part different genes, although their targets overlap considerably. Generally speaking SREBP-2 is involved in regulating genes relevant to cholesterol homeostasis, whereas SREBP-1a is implicated in fatty acid metabolism (Horton et al., 2003). Although SREBPs are required for the transcriptional activation of cholesterogenic genes, they are inefficient on their own and their full function requires the simultaneous binding of other transcription factors to regulatory elements in the vicinity of the SRE. For some genes, a single coregulatory protein is needed (Sanchez et al., 1995), while others may require more (Kim et al., 2001). For example, the transcriptional activation of the LDL receptor gene requires the presence of specificity protein 1 (Sanchez et al., 1995); the activation of the DHCR7 gene encoding for 7-dehydrocholesterol requires the simultaneous binding of specificity protein 1 and nuclear factor (Kim et al., 2001), in addition to SREBP.
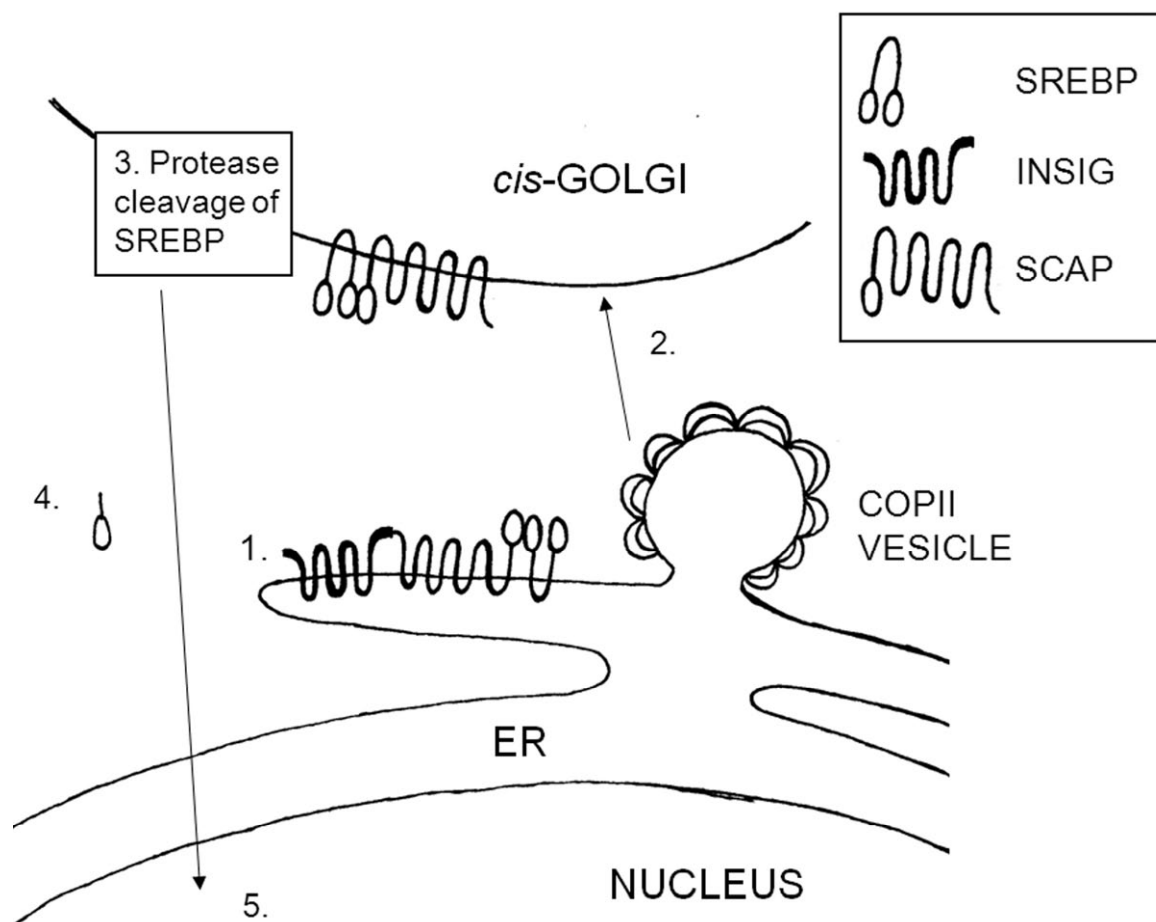
Image E. A schematic illustration of SREBP regulation. When ER sterol levels are low, SCAP escorts SREBP to the Golgi complex via COPII-coated vesicles (2.) to undergo sequential cleavage by two proteases (3.), liberating the SRE-binding fragment (4.). The fragment then enters the nucleus and modifies the expression of target genes (5.). When ER cholesterol content is high, SCAP changes conformation in a way that favors binding to the integral membrane proteins Insig-1 and Insig-2, which in turn results in the retention of the SCAP/SREBP complex in the ER (1.). Image redrawn and modified from (Ikonen, 2008).

1.2.3. Cholesterol precursors

Lanosterol is converted to cholesterol by the removal of three methyl groups, the reduction of one double bond by NADPH, and the repositioning of the other double bond (Image D). These conversions take multiple steps and are catalyzed by several enzymes. Two enzymes relevant to work presented in this thesis are now introduced further. Lanosterol undergoes 14-α-demethylation by the cytochrome P450 mono-oxygenase P45014DM, also called CYP51, encoded by the gene *CYP51* (Rozman et al., 1996). This oxygen-, heme- and NADPH-requiring step is considered to be rate-limiting in the post-lanosterol part of the cholesterol biosynthesis pathway (Frye et al., 1993). CYP51 is expressed in all eucaryotic phyla and it is ubiquitously expressed throughout different cell types. Virtually all cytochrome P450 enzymes are localized in the ER (Waterman and Lepesheva, 2005).

The immediate product of the reaction catalyzed by CYP51 is the follicular fluid meiosis activating sterol, an intermediate of cholesterol biosynthesis which normally only accumulates in testis and ovary (Byskov et al., 1995).

DHCR24 encodes for Δ24-reductase (DHCR24), the enzyme responsible for converting the penultimate cholesterol precursor, desmosterol, to the end product cholesterol (Greeve et al., 2000). In addition to its role in cholesterol biosynthesis, DHCR24 has been shown to have prosurvival effects which are mediated through several, not yet fully defined routes. DHCR24 is suggested to convey protection to oxidative and oncogenic stress via interaction with the tumor suppressor p53 and Mdm2, a negative regulator of p53 (Wu et al., 2004). These effects were found to be independent of the enzyme's reductase activity. In a recent study on neuroblastoma cells, overexpression of DHCR24 increased the cholesterol content of the cells and a prosurvival effect was conveyed in a cholesterol-dependent manner (Kuehnle et al., 2008). Mutating the reductase activity of the enzyme abolished this effect. Chronic oxidative stress decreased the levels of DHCR24 and cholesterol, and at this late stage the prosurvival effect of DHCR24 was relayed by a p53-mediated mechanism (Kuehnle et al., 2008). DHCR24 has recently been shown to be a target gene for the transcription factor LXR (Wang et al., 2008b).

In nearly all cell types the major sterol is cholesterol, and its derivatives and precursors are only present in trace amounts. Sterols which have at first glance only minor structural differences to cholesterol may differ considerably from cholesterol in their membrane partitioning, trafficking and metabolic properties. For example desmosterol, which is the penultimate precursor of cholesterol and only differs from the end product by a single double bond, cannot fully replicate the biological functions of cholesterol in membranes, as evidenced by its compromised ability to support raft-dependent insulin signaling (Vainio et al., 2006). Regardless, cholesterol is not the only major sterol present in the membranes of some cells. High concentrations of desmosterol are found in primate spermatozoa and in tissues of the male reproductive tract of various species (Lindenthal et al., 2001). Desmosterol is also present in high concentrations in the developing brain (Fumagalli and Paoletti, 1963) where it accumulates just prior to myelination (Hinse and Shah, 1971). As of yet, no specific physiological function has been identified for desmosterol or other sterol precursors in the CNS.

Inborn errors of metabolism which lead to the accumulation of a precursor sterol and diminished or abolished cholesterol synthesis cause severe congenital syndromes affecting many parts of the body (Hennekam, 2005). Human disorders involving several steps of post-squalene cholesterol biosynthesis have been described. A shared feature of these disorders is dysmorphogenesis of multiple organs, including the CNS (Moebius et al., 2000). Defects in cholesterol synthesis can in principle cause malformations by two mechanisms: the lack of cholesterol or the accumulation of sterol intermediate(s). It is assumed that many of the malformations present in genetic defects of cholesterol biosynthesis can be attributed to perturbations in Hedgehog signaling. For example, 7-dehydrocholesterol, which is the accumulating sterol in Smith-Lemli-Opitz syndrome (Moebius et al., 2000), can not fully stimulate the Sonic hedgehog signaling pathway. The autoprocessing of Sonic hedgehog can be completed in Smith-Lemli-Opitz cells

maintained in low-cholesterol conditions, but the ability of the cells to respond to Sonic hedgehog signaling is severly compromised (Cooper et al., 2003). This is likely to be due to lack of cholesterol, as the same was found to be true for cells with no sterol biosynthesis defect when cholesterol was depleted with methyl-β-cyclodextrin treatment (Cooper et al., 2003). The paucity of cholesterol or the substitution of other sterols may also affect several other sterol-sensing proteins, such as SCAP and NPC1 (Porter, 2002).

Evidence for a role for the accumulating sterols themselves in the pathogenesis of these disorders was uncovered when the hallmarks of these syndromes were reproduced in Insig-1/Insig-2 double knockout mice (Engelking et al., 2006). These mice have constitutively upregulated sterol synthesis accompanied by accumulation of sterol intermediates. The midline defects could be alleviated by blocking cholesterol synthesis with lovastatin, suggesting that the accumulation of intermediates, not the lack of cholesterol, was responsible for the phenotype (Engelking et al., 2006). Accumulating sterol intermediates may influence morphogenesis and cell function in other ways, for example by modulating membrane properties and thereby affecting the activation of signaling pathways.

1.3. How cells handle excess cholesterol

Excess free cholesterol is toxic to cells (Yao and Tabas, 2001). There is no known mechanism for cells to degrade sterols and any amount of cholesterol exceeding the cells' needs must be excreted or stored in lipid droplets. The storage form of cholesterol is CE, in which a fatty acyl moiety is linked to the hydroxyl group of cholesterol via an ester bond. Free cholesterol is thought to be esterified in the ER by the acyl coenzyme A:cholesterol acyltransferase (ACAT) enzymes (Chang et al., 2001). The human *ACAT1* was the first ACAT gene to be identified (Chang et al., 1993). It is expressed in a wide variety of cells, notably in macrophages (Lee et al., 2000). However, a mouse model of *Acat1* deficiency did not exhibit diminished cholesterol esterification in the liver. Additionally, intestinal cholesterol absorption was not impaired (Meiner et al., 1996). The disruption of ACAT activity was expected to impair cholesterol absorption in these mice as ACAT activity is required to esterify cholesterol for packaging into nascent chylomicrons (Temel et al., 2005). ACAT2 was subsequently identified based on C-terminal protein sequence homology with ACAT1 (Anderson et al., 1998).

Excess cholesterol can be removed from the cell in a process termed reverse cholesterol transport. In tissues outside of the CNS, sterol removed from cells is incorporated into high-density lipoprotein (HDL) particles and transported in the bloodstream to be taken up by hepatocytes and to a lesser extent, steroidogenic cells (Van Eck et al., 2005). Initially, lipid-poor apolipoproteins, such as ApoAI, are lipidated by the ATP-binding cassette transporter ABCA1 (Zannis et al., 2006). The lipidation of ApoAI leads to the formation of discoidal pre-β-HDL particles (Hara and Yokoyama, 1991), which are modified and lipidated further in the blood by the lechithin-cholesterol acyl transferase (LCAT) (Zannis et al., 2006). Mutations in the gene encoding ABCA1 lead to Tangier disease (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Rust et al., 1999),

which is characterized by a virtual absence of plasma HDL particles and the deposition of cholesteryl esters in the reticuloendothelial system.

In target cells, HDL apolipoproteins are bound by class B scavenger receptors (SR-BI) (Acton et al., 1996). Unlike in the receptor-mediated endocytosis of LDL particles, only the cholesteryl esters are taken up from the lipidated HDL particles by SR-BI in a process termed selective lipid uptake (Rinninger and Pittman, 1987). Cholesteryl esters taken up via this route are hydrolysed extralysosomally by a neutral hydrolase (Shimada et al., 1994). SR-BI is bi-directional in its function and also facilitates the efflux of cholesterol to extracellular acceptors (Yancey et al., 2000). According to some reports, SR-BI expression is not under LXR or SREBP regulation (Yu et al., 2004). However, a recent study shows a transcriptional down-regulation of SR-BI in mice constituitively overexpressing SREBP-1a or -1c (Niemeier et al., 2008).

Oxidized derivatives of sterols, oxysterols, are present in cells in low amounts and are potent modulators of sterol metabolism (Kandutsch et al., 1978). They can be produced via auto-oxidation of cholesterol derived from food, or endogenously via enzymatic conversion (Bjorkhem and Diczfalusy, 2002). Endogenous oxidation may also happen non-enzymatically through the production of reactive intermediates generated in the course of lipid peroxidation (Vaya and Schipper, 2007). In general, the oxygenation of a sterol drastically reduces its half-life, enhances its ability to cross biological membranes and directs it to excretion or further oxidation to bile acids (Bjorkhem and Diczfalusy, 2002). LXR α and β are members of the nuclear receptor family of transcription factors. They bind oxysterols and mediate their effects on cholesterol balance by modifying the transcription of genes associated with lipid metabolism (Lehmann et al., 1997). These include several ATP-binding cassette (ABC) proteins implicated in cholesterol removal from cells, apolipoprotein E (ApoE) and other lipoproteins, and genes encoding lipoprotein modifying enzymes (Tontonoz and Mangelsdorf, 2003). In addition to LXRs, the farnesoid X receptor (FXR) also binds to and is activated by oxysterols (Bjorkhem and Diczfalusy, 2002). The function of FXR is vital to bile acid homeostasis (Makishima et al., 1999).

In order to exert their effects on transcriptional activation, LXRs and FXR must form heterodimers with the retinoid X receptor (RXR) (Mangelsdorf and Evans, 1995). The identity of the physiological ligands of these receptors has been controversial (Bjorkhem and Diczfalusy, 2002). The role of oxysterols as physiological LXR ligands was recently confirmed by demonstrating the inability of dietary cholesterol to regulate gene expression in a mouse knockout model incapable of producing 24S-hydroxycholesterol, 25-hydroxycholesterol and 27-hydroxycholesterol (Chen et al., 2007). As further proof, the authors demonstrated that cholesterol sulfotransferase, an enzyme capable of metabolizing oxysterols, inactivates LXR signaling when expressed in cultured cells. The cytosolic oxysterol binding protein (OSBP) and its homologues may also play a role in mediating the effects of oxysterols (Olkkonen, 2004). In addition to their regulatory roles, oxysterols serve as precursors for bile acid synthesis and function as a transportable form of sterol, thus playing a role in the efflux and removal of sterols (Lutjohann et al., 1996).

2. Cholesterol in the Central Nervous System

The CNS is the most cholesterol-rich organ in the body. In humans, the brain comprises circa 2% of body weight, but an estimated 25% of the total free cholesterol in the body is found in the CNS (Dietschy and Turley, 2001). The mechanisms of cholesterol metabolism in the CNS are rather poorly understood compared to our knowledge of peripheral cholesterol homeostasis. It has long been recognized that significant differences exist in cholesterol metabolism between the CNS and the periphery. A blood-brain barrier (BBB) formed by capillary endothelial cells and specialized cells of the CNS isolates the CNS from peripheral circulation and interstitial fluid. Lipid-soluble molecules cross the BBB by diffusion, but carrier-mediated transport is required to enable molecules with low lipid solubility to cross. Importantly, it is thought that cholesterol-carrying blood lipoproteins do not enter the CNS through the BBB (Chobanian and Hollander, 1962) even in the embryo (Plotz et al., 1968; Tint et al., 2006). However, ventricular zone neural progenitor cells have recently been shown to survive without *de novo* cholesterol synthesis, possibly due to ability to take up cholesterol-bearing lipoproteins from the ventricular fluid or from the blood (Saito et al., 2009). All or nearly all cholesterol in the CNS is thought to derive from *de novo* synthesis. Essentially all cholesterol in the healthy CNS is in the unesterified form (Bjorkhem and Meaney, 2004), and the expression of ACAT is low (Uelmen et al., 1995).

2.1. Roles of cholesterol in the CNS

In mature CNS tissue, most cholesterol is present in the myelin membranes. This mass of comparatively inert membrane cholesterol masks more subtle changes in sterol metabolism when analyzed with biochemical techniques and, for decades, the prevailing view was that the CNS is an inactive and "uninteresting" organ when it comes to cholesterol metabolism (Dietschy and Turley, 2001). Interest in the field started to rise as key discoveries pointing toward the importance of CNS cholesterol in health and disease were made, for example the discovery of the *APOE* ε4 allele as a risk factor for late-onset sporadic Alzheimer's disease (Corder et al., 1993) and the identification of cholesterol as a glia-derived synaptogenetic factor (Mauch et al., 2001).

Essential neuronal functions rely on the availability of cholesterol and the integrity and function of membranes. Cholesterol is essential for maintaining an intact plasma membrane, which is required for maintaining the ion environment necessary for the generation and propagation of action potentials. Synaptic function depends on cholesterol in many ways (Pfrieger, 2003a), as discussed in more detail later. The availability of cholesterol has been shown to be critical for the morphogenesis of the CNS during development (Engelking et al., 2006). Cholesterol is also utilized as a precursor in the synthesis of CNS-produced neuroactive steroids, neurosteroids (Melcangi et al., 2008). Myelin is rich in cholesterol as well as other lipids (Saher et al., 2005). Loss of myelin leads to neuronal dysfunction, as evidenced by demyelination in several neurological disorders, for example multiple sclerosis (Genain et al., 1999). Defects in cholesterol

biosynthesis have been described in various neurodegenerative diseases. A recent example is Huntington's disease, a progressive autosomal dominant neurodegenerative disease of adulthood. In the study by Valenza and co-workers (Valenza et al., 2005), cells harbouring a mutant huntingtin were shown to have impaired SREBP translocation to the nucleaus and a markedly lower rate of cholesterol synthesis than control cells when cultured in lipid-poor conditions. Adding cholesterol to neurons transfected with a mutant huntingtin rescued them from cell death, highlighting the importance of cholesterol dysfunction in this disease model.

2.2. Cells of the CNS

The nervous system is composed of nerve cells, or neurons, and various types of specialized support cells which are collectively called glial cells. CNS glial cells are further divided into astrocytes, oligodendrocytes and microglia (Fields and Stevens-Graham, 2002).

Astrocytes are intimately associated with neuronal synapses and have been implicated in a variety of functions: connective tissue functions, participating in the formation of the blood-brain barrier, taking part in repair and recycling events in the CNS, and several others (Siegel et al., 1999). They are the source of the majority of CNS lipoproteins (Xu et al., 2006) and considered central to CNS sterol metabolism. They have been shown to provide neurons with cholesterol that is necessary during synapse formation (Mauch et al., 2001) and participate in the recycling of cholesterol after injury (Danik et al., 1999). It has been suggested that mature neurons rely on astrocyte-derived cholesterol *in vivo*, as they synthesize sterols inefficiently as compared to astrocytes (Nieweg et al., 2009).

Oligodendrocytes form the myelin sheath which envelops the axons of many neurons in vertebrates. They wrap their own plasma membranes tightly around the axons to insulate the axonal membrane so little current can leak across, thereby making the propagation of action potentials more efficient (Fields and Stevens-Graham, 2002). Microglial cells are CNS cells derived from bone marrow monocyte precursors (Fields and Stevens-Graham, 2002). They are thought to become mobile macrophage-like cells upon activation, and play a central role in CNS immune responses.

A fourth non-neuronal cell type, the ependymal cell, is found lining the ventricles and the spinal canal (Siegel et al., 1999). The ependyma is a cuboidal to columnar, ciliated epithelium, which has considerable metabolic activity as well as barrier functions (Del Bigio, 1995). Specialized ependymal cells also participate in the formation of cerebrospinal fluid. Ependymal cells of the spinal cord harbor *in vitro* neural stem cell activity and can give rise to scar-forming astrocytes and remyelinating oligodendrocytes in response to injury (Meletis et al., 2008). Adult forebrain ependymal cells are quiescent under normal conditions, but activate after stroke to produce neuroblasts and glial cells (Carlen et al., 2009).

The bulk of cholesterol in the mature CNS is found in myelin (Dietschy and Turley, 2001). Most cholesterol in this pool appears to be synthesized by the myelin-forming oligodendrocytes, as hypomyelination occurs in mice with a conditional loss-of-function

mutation of the gene encoding for squalene synthase in these cells (Saher et al., 2005). Some transfer of cholesterol between cell populations seems possible however, as the phenotype of the hypomyelinated mice was found to be alleviated with age (Saher et al., 2005). Once myelination is completed, the turnover of cholesterol in this pool is considered to be very slow compared to gray matter (O'Brien, 1965). Another pool of CNS cholesterol is formed by the membranes of neurons and glial cells other than oligodendrocytes. The turnover of cholesterol in the adult CNS overall is slow compared to peripheral tissues. The rate of cholesterol flux across the CNS is estimated at 0.9% of the rate of cholesterol turnover in the rest of the body (Dietschy and Turley, 2001).

2.3. Cholesterol and the neuron

Signaling between nerve cells, and sometimes neurons and non-neuronal cells, occurs via synapses. Synapses seem to be relatively enriched in cholesterol, as the cholesterol/phospholipid ratio in synaptosome preparations is similar to that of the plasma membrane (Pfrieger, 2003a). However, synaptosome preparations inevitably contain postsynaptic material, so the actual cholesterol content in the synaptic terminal may be higher. In the CNS, availability of astrocyte-derived cholesterol is a prerequisite for synaptogenesis (Mauch et al., 2001). Although cholesterol seems to be required, it may not be sufficient on its own to support the formation of functional synapses (Christopherson et al., 2005). Cholesterol has been found to be the most abundant lipid in synaptic vesicles (SV), accounting for circa 40% of lipid in these structures (Takamori et al., 2006).

Key synaptic functions have been found to be cholesterol-dependent. Cholesterol is required for the normal function of neurotransmitter receptors, as for instance the nicotinic acetylcholine receptor has been shown to embed cholesterol and to require it for functionality (Brannigan et al., 2008). These receptors belonging into the Cys-loop superfamily of ion channels mediate transmission in the central and peripheral nervous systems. They are widely expressed and associated with diseases such as Alzheimer's disease, Parkinson's disease and neuropathic pain. These channels form ligand-gated ion channels and do not make use of a second messenger (Itier and Bertrand, 2001).

Cholesterol is suggested to be necessary for the formation, transport and function of SVs. The axonal kinesin-mediated membrane traffic along microtubules requires cholesterol-rich rafts (Klopfenstein et al., 2002). The biogenesis and exocytosis of synaptic-like microvesicles in PC12 cells, a neuroblastoma cell line, has been shown to be cholesterol-dependent (Thiele et al., 2000). This phenomenon was interpreted as specific to synaptic-like microvesicles, as Thiele and colleagues could not observe a major impairment in total endocytotic activity. The cholesterol content in SV membranes may also play a part in neurotransmitter release, as release of presynaptic neurotransmitters requires the fusion of SV membranes with the membrane of the presynaptic terminal. Synaptobrevin, also called VAMP (short for vesicle-associated membrane protein), is one of the soluble *N*-ethylmaleimide-sensitive attachment protein receptor (SNARE) proteins required for the assembly of SNARE complexes (Sollner et al., 1993). SNARE complexes

mediate in membrane fusion (Sollner et al., 1993), a key event in neurotransmitter release from SVs. Synaptophysin interacts with synaptobrevin in a cholesterol-dependent manner (Mitter et al., 2003). Synaptophysin has been speculated to participate in the control of synaptobrevin function, as synaptobrevin interaction with synaptophysin is exclusive of its interaction with its SNARE partners (Edelmann et al., 1995). However, the presence of synaptophysin did not exert any measurable effect on the formation of SNARE complexes as reported by Siddiqui and co-workers (Siddiqui et al., 2007), and a knock-out mouse model failed to demonstrate an essential role for synaptophysin (McMahon et al., 1996). Several functions have been suggested for synaptophysin, including a role in SV biogenesis by participating in the cholesterol-dependent formation of high curvature membranes (Thiele et al., 2000), but as of now its function remains uncertain.

During development and nerve regeneration, axonal growth is guided by extracellular attractive and repulsive cues, which influence the directional motility of axonal growth cones. Cholesterol-rich lipid rafts have been suggested to mediate guidance responses in nerve growth cones. A study by Guirland and colleagues (Guirland et al., 2004) demonstrated that disruption of lipid rafts with methyl-β-cyclodextrin specifically interferes with Xenopus growth cone guidance by gradients of brain derived neurotrophic factor, netrin-1 or Semaphorin 3A without affecting axon length or growth cone guidance by glutamate. The receptors for the guidance cues were found to be weakly associated with rafts under baseline conditions, but after stimulation with their respective ligands, the receptor-raft association was found to be markedly enhanced. The raft-association of the netrin-1 receptor DCC was confirmed in mammalian cells by another group (Herincs et al., 2005). This study also showed that netrin-1-mediated DCC-dependent commissural axon outgrowth requires lipid raft integrity in mammalian peripheral nervous system neurons, and that netrin-1 mediated activation of ERK1/2 signaling is dependent on DCC association with lipid rafts. Cholesterol modulation was also shown to influence the maturation of hippocampal and cortical neurons *in vitro* via a Fyn signaling-mediated mechanism (Ko et al., 2005).

It seems apparent that the availability of cholesterol is critical for numerous functions in the CNS. What is known about the mechanisms governing cholesterol metabolism in the CNS?

2.4. Sterol metabolism in the CNS

In addition to the isolation of the CNS from peripheral lipoproteins, other factors indicate functional differences between the mechanisms of sterol metabolism in the CNS and the rest of the body. Some proteins central to cholesterol homeostasis in peripheral tissues are not found in the CNS, or their expression patterns are different. For example, the nuclear receptor LXRα is expressed at high levels in peripheral organs involved in lipid homeostasis such as liver, intestine, and brown adipose tissue, whereas LXRβ is enriched in neuronal tissues and tissues of endocrine origin (Annicotte et al., 2004). Another example is ACAT, which is only expressed at low levels in the CNS (Uelmen et al.,

1995). Not all cholesterol-related proteins are expressed by all CNS cell types. For example, sterol 24-hydroxylase, an enzyme involved in sterol egress from the CNS, is mostly found in neurons (Lund et al., 1999) while ApoE is expressed by astrocytes (Pitas et al., 1987b). This may reflect a division of labour between the different cell populations in the CNS. An astrocyte-neuron cholesterol "shuttle" system has been suggested (Pfrieger, 2003b), where mature neurons rely on cholesterol produced by astrocytes under normal conditions. Indeed, mature cerebellar neurons with a conditional knock-out of squalene synthase have been shown to survive *in vivo*, lending support to this theory (Funfschilling et al., 2007). Moreover, the patterns of protein expression between the cell populations are not static, but may change during development (Husemann and Silverstein, 2001) or injury (Laskowitz et al., 1998).

Cholesterol turnover, albeit slow in the normal CNS, is required for essential brain functions such as learning (Kotti et al., 2006). Conversion of cholesterol to 24S-hydroxycholesterol (cerebrosterol) is a major route of cholesterol clearance in the CNS (Lutjohann et al., 1996). *CYP46* encodes for cholesterol 24-hydroxylase, a cytochrome P450 enzyme responsible for this conversion (Lund et al., 1999). Unlike cholesterol, 24S-hydroxycholesterol is able to cross the BBB by diffusion. After entering the bloodstream, it is transported to the liver for clearance (Bjorkhem et al., 1998). 24S-hydroxycholesterol is synthesized in the CNS at a rate of at least half of the rate of cholesterol synthesis (Bjorkhem et al., 1997). 24-hydroxylase is found in neurons throughout the brain, but thought to be absent from glial cells (Lund et al., 1999; Karasinska et al., 2009). However, a recent report shows co-localization of 24-hydroxylase with the astrocyte marker GFAP in the hippocampi of wild-type mice (Karasinska et al., 2009).

The rate of CNS cholesterol synthesis is closely coupled to the rate of cholesterol clearance via cholesterol-24-hydroxylase, as *Cyp46* knockout mice display a tissue-selective 40% reduction in the rate of cholesterol synthesis in the brain (Lund et al., 2003). These knockout mice are viable and display no obvious anomalies. The brain also contains a sterol 27-hydroxylase (Pedersen et al., 1989) but its activity is lower than that of 24-hydroxylase. 27-hydroxylase activity may account for a minor elimination route of cholesterol from the CNS (Bjorkhem et al., 1997). Conversion to 24S-hydroxycholesterol accounts for a major part of cholesterol clearance from the CNS, but as the rate of input from cholesterol synthesis is greater than output through conversion to 24S-hydroxycholesterol or other more minor identified oxysterol species, as of yet unidentified routes of elimination probably exist (Bjorkhem et al., 1997). It is noteworthy that *Cyp46* knockout animals have a reduced, but not abolished, rate of cholesterol synthesis, supporting the notion that alternative pathways for cholesterol clearance must exist. 24S-hydroxycholesterol is likely to have regulatory roles as well. It has been shown to be a potent down-regulator of cholesterol synthesis (Saucier et al., 1989), and it has a high affinity for LXRα and LXRβ (Janowski et al., 1999). It is possible that 24S-hydroxycholesterol functions as a feedback regulator of cholesterol amount in CNS cells.

2.5. Lipoproteins in the CNS

The lipoprotein system in the brain is distinct from the periphery. The main apolipoprotein in the CNS is ApoE, wheras ApoB, the main apolipoprotein of peripheral LDL-particles, is absent (Pitas et al., 1987a). Under normal conditions, astrocytes are thought to produce most of the ApoE-containing, HDL-like lipoprotein particles found in the CNS (Pitas et al., 1987b). In a mouse model expressing EGFP as a reporter under the ApoE promoter, hippocampal neurons and microglia did not express ApoE, but it was expressed by most astrocytes (Xu et al., 2006). All of the ApoE found in cerebrospinal fluid (CSF) is produced in the CNS, as the ApoE phenotype of CSF lipoproteins does not change in liver transplant recipients (Linton et al., 1991). In addition to astrocytes, microglia have been shown to secrete lipoproteins *in vitro*, but in these particles ApoJ is the major apolipoprotein (Xu et al., 2000). ApoJ, also called clusterin, is a glycoprotein with a propensity to interact with a wide variety of molecules. Several functions have been attributed to it, including participation in death signaling, lipid trafficking and chaperone functions, but no clear consensus has been found (Jones and Jomary, 2002). In human plasma, ApoJ is associated with HDL-particles (de Silva et al., 1990). Unlike ApoE, ApoJ is readily transported across the BBB via receptor-mediated transcytosis through its interaction with Megalin (Zlokovic et al., 1996).

ApoAI, ApoAIV and ApoD are minor lipoprotein species found in the CNS or in the CSF (Harr et al., 1996). ApoAI is present in CSF (Pitas et al., 1987a), but it is thought to be derived from outside of the CNS as no ApoAI mRNA could be detected in the CNS (Elshourbagy et al., 1985). However, ApoAI has been reported to be found in human brain parenchyma as determined by Western blotting (Harr et al., 1996; Huang et al., 2007). Its role in CNS sterol metabolism, if any, is uncertain. ApoAI is also expressed by brain capillary endothelial cells (Mockel et al., 1994), so determination of ApoAI protein from intact tissue may represent ApoAI expressed in these cells or even contaminating protein originating from peripheral blood trapped in capillaries (Boyles et al., 1985). Apolipoprotein D (ApoD) has originally been identified as a constituent of plasma HDL (McConathy and Alaupovic, 1973). It belongs to the lipocalin superfamily of small hydrophobic transport proteins (Drayna et al., 1986) and bears little homology to other apolipoproteins. It is expressed in the fetal CNS (Drayna et al., 1986), but its physiological function in the brain remains unclear. It has been reported to bind steroid hormones and arachidonic acid, but not cholesterol (Morais Cabral et al., 1995). ApoAIV is a 46kDa protein associated with HDL in the blood. In the brain, ApoAIV may function as a satiation protein. It is produced in response to fat feeding in the small intestine and neurons of several key brain areas important for energy homeostasis regulation (Shen et al., 2008).

The lipoprotein particles secreted by astrocytes *in vitro* are heterogenous in size and shape. They have been descibed to be similar in size to plasma pre-β-HDL-like particles and discoidal or spherical in shape (LaDu et al., 1998; Fagan et al., 1999; DeMattos et al., 2001). They contain mainly ApoE as their apolipoprotein, but ApoJ has also been reported (LaDu et al., 1998). Astrocyte-secreted lipoproteins contain free cholesterol and phospholipids, but little core lipid (LaDu et al., 1998; DeMattos et al., 2001). The ApoE-

containing lipoprotein particles secreted by astrocytes are lipidated by transporters in the ABC-family, such as ABCA1 (Wahrle et al., 2004). ABCA1-independent lipidation routes may exist as ABCA1-deficient cells are still capable of facilitating cholesterol and ApoE efflux, although the efflux is less efficient than in wild-type cells (Hirsch-Reinshagen et al., 2004).

ABCG1 and ABCG4 are ATP-binding cassette proteins which are highly expressed in the brain (Tachikawa et al., 2005). Overexpression of these proteins in cultured human embryonic kidney cells can mediate cholesterol transfer to HDL particles but not lipid-poor ApoA-I (Wang et al., 2004). They have been shown to mediate the efflux of cholesterol and desmosterol to HDL particles from primary astrocytes (Wang et al., 2008a). In primary astrocytes prepared from mice deficient in either or both of the proteins, cholesterol and desmosterol accumulate and sterol synthesis is down-regulated (Wang et al., 2008a).

Several members of the LDL receptor family are expressed in the CNS, including LDLR itself (Hanaka et al., 2000), LDL-receptor related protein (LRP1) (Ishiguro et al., 1995), Megalin (Aglyamova and Agarwala, 2007), VLDL receptor (VLDLR) (Christie et al., 1996) and ApoE receptor 2 (ApoER2) (Stockinger et al., 1998). They all bind ApoE, but LRP1, Megalin, VLDLR and ApoER2 also bind a variety of other ligands (Herz and Bock, 2002). Receptor associated protein (RAP) is a chaperone which binds newly synthesized receptors and prevents their association with ligands until they reach the cell surface (Willnow, 1998). The neurite-growth-promoting effects of CNS ApoE-containing lipoprotein particles can be blocked to a large extent by the presence of RAP or antibodies to LRP1, suggesting that LRP1 is the main transport protein involved in mediating these effects (Fagan et al., 1996). LRP1 has also been directly implicated in synaptic plasticity in hippocampal (HC) slices, as RAP was able to block long term potentiation in hippocampal neurons in this model (Zhuo et al., 2000). VLDLR and ApoER2 function as receptors for Reelin, a regulator of neuronal migration (D'Arcangelo et al., 1999).

Megalin, also called glycoprotein 330, is the only identified receptor for ApoJ (Kounnas et al., 1995). It is expressed on the ependymal cells lining the ventricles in the mature brain, but not in the brain parenchyma (Chun et al., 1999). Megalin also functions as a Hedgehog receptor (McCarthy et al., 2002) and during development it is expressed in the ventral midbrain and tectum (Aglyamova and Agarwala, 2007). The level of SR-BI in the CNS is low (Acton et al., 1996), but it has been shown to be expressed by astrocytes in the adult brain (Husemann and Silverstein, 2001). LR11/SORLA is found in the CNS (Herz and Bock, 2002) and it has been shown to bind ApoE (Yamazaki et al., 1996). Its function is not conclusively established, but in addition it is a member of the LDL receptor family and also belongs the vacuolar protein sorting 10 protein (VPS10p) family of intracellular sorting receptors (Herz and Bock, 2002). LRP6 has been implicated in LDL clearance (Liu et al., 2008) and its expression has been described in the CNS (Herz and Bock, 2002). In addition, several other members of the LDL receptor family have been found in the CNS, id est LRP1b, LRP5, MEGF7, but their functions may not be linked to cholesterol metabolism (Herz and Bock, 2002).

Evidence from knock-out animals points to flexibility and redundancies in the CNS lipoprotein system. Mice lacking LDLR have normal brain development (Herz and Bock,

2002). *Apoe* knockout mice are utilized extensively as a model for atherosclerosis. They are viable and have no gross abnormalities (Zhang et al., 1992). *Abca1* floxed, nestin-Cre positive mice which lack *Abca1* expression in the CNS are viable, fertile and show no gross abnormalities (Karasinska et al., 2009). In contrast, LRP1 is essential to normal embryo development and a conventional knockout of *Lrp1* leads to early embryonic lethality (Herz et al., 1992). Mice with cre-loxP-mediated disruption of the *Lrp1* gene in postmitotic differentiated neurons appear normal at birth, are viable and have no developmental defects in their CNS apparent upon histological examination, but suffer from behavioural and motor abnormalities (May et al., 2004). In contrast, *Megalin* knockout mice are born with holoprocencephaly (Willnow et al., 1996), highlighting the importance of Megalin in early CNS development.

3. Cholesterol in neurodegeneration

3.1. Niemann-Pick disease type C

Niemann-Pick disease type C (NPC) is a rare, recessively inherited, fatal neurodegenerative disorder. The age of onset varies from infancy to childhood, but in all cases the cellular phenotype is characterized by the intracellular accumulation of cholesterol and sphingomyelin in the late endosomes/lysosomes. Symptoms include dystonia, ataxia and progressive dementia (Patterson, 2003; Ikonen and Holtta-Vuori, 2004). The prevalence of NPC has been estimated to be 1:150000 in Western Europe, but this may represent an underestimate due to the limited availability of biochemical testing to obtain definite diagnosis (Vanier and Millat, 2003). The prevalence can be much higher in some genetic isolates, for example in Nova Scotia, where NPC is found in the descendants of one founder couple (Winsor and Welch, 1978).

NPC is caused by mutations in one of two genes, *NPC1* or *NPC2*. The gene responsible for the majority of cases, *NPC1*, was the first to be identified (Carstea et al., 1997). Mutations in *NPC2* are causative in a minor subgroup of cases (Naureckiene et al., 2000). NPC1 is a multi-membrane span protein, which resides in late endosomes. Five of the transmembrane domains of NPC1 constitute a sterol-sensing domain (SSD), which shares homology with SSDs found in the Hedgehog receptor Patched, HMG-CoAR, SCAP and SREPB (Carstea et al., 1997). The SCAP SSD is known to be involved in the binding of SCAP to Insig (Yabe et al., 2002). Transmembrane protein 97 (TMEM97) was recently identified as a binding partner for NPC1 (Bartz et al., 2009), but exact nature of this interaction remains to be elucidated. NPC2 is a small, secretory glycoprotein with cholesterol-binding properties, which is targeted to lysosomes via the mannose-6-phosphate receptor (M6PR) pathway (Naureckiene et al., 2000; Willenborg et al., 2005). Cells lacking M6PRs have been shown to accumulate cholesterol in late endosomes in the same manner as NPC cells, suggesting that M6PR binding to NPC2 is important in cholesterol transport (Reaves et al., 2000). The Nogo-B receptor (NgBR) was recently

identified as an extralysosomal interaction partner for NPC2 (Harrison et al., 2009). The authors demonstrated that the lack of NgBR leads to an NPC-like phenotype in cultured cells, underlining the potential importance of this interaction in NPC2 function.

NPC cells accumulate sphingomyelin and LDL-derived free cholesterol in their late endosomes, as they are unable to facilitate lipid egress from these organelles. Consequentially, cholesterol does not reach the ER and the mechanisms regulating cholesterol metabolism are disturbed. The transport of newly synthesized cholesterol to the plasma membrane is independent of NPC1 (Liscum et al., 1989), but newly synthesized cholesterol is also eventually trapped in the endosomal system of NPC cells (Cruz and Chang, 2000). The esterification of cholesterol for storage in NPC cells is low although ACAT activity is not impaired (Pentchev et al., 1985). The feedback regulation of HMG-CoAR activity and LDLR expression mediated by LDL-derived cholesterol is also impaired (Liscum and Faust, 1987). Essentially, the disease cells respond as though they lack cholesterol in spite of the lysosomal accumulation.

Despite intense effort, the exact mechanisms of function of the NPC proteins have not been elucidated. NPC1 has been suggested to participate in the movement and partitioning of endosomes (Ko et al., 2001), and function as a fatty acid permease (Davies et al., 2000), whereas NPC2 has been suggested to function as a cholesterol shuttle (Cheruku et al., 2006). The clinical syndrome and biochemical features caused by mutations in either *NPC1* or *NPC2* are identical, and the proteins have been suggested to function in concert (Naureckiene et al., 2000; Infante et al., 2008). The high-resolution structure of the N-terminal domain of NPC1 was recently elucidated (Kwon et al., 2009). The authors propose a working model, in which LDL-derived cholesterol binds NPC2 within lysosomes. NPC2 then transfers the cholesterol to NPC1, which in turn reverses the orientation of cholesterol and allows its insertion into the membrane. ABCA1-dependent efflux of cholesterol to ApoAI has been shown to be compromised in NPC1-deficient cells (Choi et al., 2003). NPC1-deficient cells also have a diminished availability of both LDL-derived and steady state cholesterol to methyl-β-cyclodextrin, a synthetic acceptor, reflecting the inability of LDL-derived cholesterol to reach the PM normally (Lusa et al., 2001).

In the brain, NPC causes the accumulation of lipids in virtually every cell type, but not all cells are equally affected. Compared to controls, there is a marked death of cerebellar Purkinje cells, a less substantial loss of neurons in the thalamus and prefrontal cortex and demyelination of the corpus callosum (German et al., 2001). The clinical presentation of the disease is highly variable and has no direct correlation to the severity of the biochemical findings (Ikonen and Holtta-Vuori, 2004). Interestingly, neurofibrillary tangles immunologically identical to those in Alzheimer's disease have been observed in the brains of NPC patients (Distl et al., 2003).
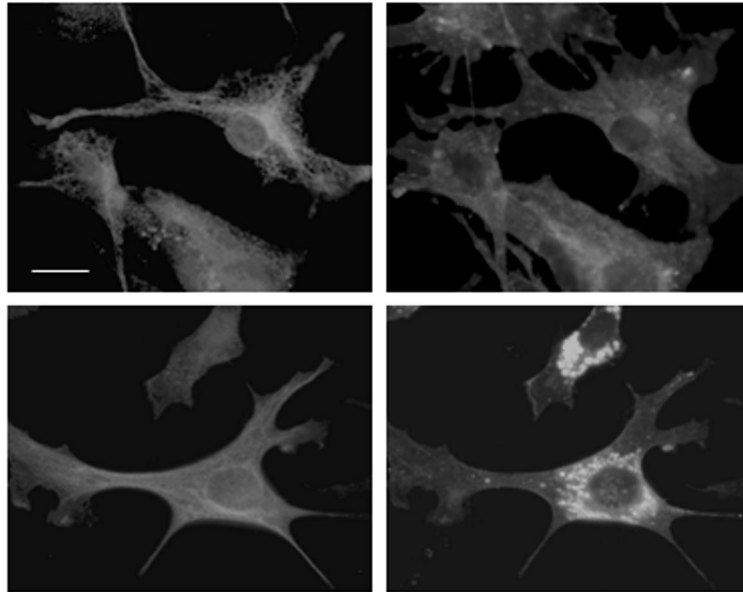
Image F: Filipin-positive cholesterol accumulation in NPC1$^{-/-}$ astrocytes. On the left: staining with antibodies for GFAP, an astrocyte marker. On the right: filipin staining. Upper panels: wild type cells. Lower panels: NPC1 knock out cells. This research was originally published in I. © The American Society for Biochemistry and Molecular Biology.

A naturally-occurring mouse model for NPC1 has been identified. This mouse strain carries a mutation in the *Npc1* gene which leads to a progressive neurodegenerative disease resembling that seen in human NPC patients. In the mutant mouse *Npc1* cDNA, 44 base pairs of WT sequence are deleted and replaced by 24 base pairs from a mammalian apparent long terminal repeat retrotransposon, resulting in a stop codon and premature truncation of NPC1 (Loftus et al., 1997). CNS specific expression of wild type *Npc1* under the prion promoter was able to restore the fertility and lifespan of *Npc1* deficient mice (Loftus et al., 2002), highlighting the importance of CNS involvement in the pathogenesis of NPC.

3.2. Infantile neuronal ceroid lipofuscinosis

Infantile neuronal ceroid lipofuscinosis (INCL) is a rare, recessively inherited disease which is considered to belong to the so-called Finnish disease heritage. An estimated 1 in 70 carry the disease gene in the Finnish population (Syvanen et al., 1997). Symptoms usually appear in infancy and lead to death in the early teenage years (Santavuori et al., 1973). Rarely, if residual activity of the dysfunctional enzyme is present, the age of onset can also be significantly later and the disease can even present in adulthood (Ramadan et al., 2007). No specific treatment is available for INCL.

INCL is one of a group of several recessively inherited neurodegenerative diseases, the neuronal ceroid-lipofuscinoses (NCL). The NCL diseases are storage disorders characterized by the lysosomal accumulation of autofluorescent material. In most NCL diseases, the autofluorescent storage material consists mainly of subunit C of ATP

synthase, but in INCL the accumulating material is mainly sphingolipid activator proteins A and D, also called saposins (Tyynela et al., 1993). This accumulation is accompanied by progressive and selective loss of cortical and cerebellar neurons, leading to neurological symptoms such as visual impairment, mental and motor deterioration and ultimately premature death (Haltia, 2006). There is considerable genetic heterogeneity underlying the NCL diseases. Several mutations in nine genes have been identified to date as causes of these disorders (Haltia, 2006; Siintola et al., 2007). An additional candidate NCL disease gene, *CLCN6*, has been suggested (Poet et al., 2006). The underlying pathogenetic mechanisms remain elusive.

INCL (also called CLN1) is caused by mutations in the *PPT1* gene (Vesa et al., 1995). These mutations abolish the activity of palmitoyl-protein thioesterase-1 (Ppt1). The physiological substrate(s) of Ppt1 are unknown, but it has been shown to be able to remove palmitic acid from S-acylated proteins *in vitro* (Camp and Hofmann, 1993). S-acylation is a post-translational modification in which a fatty acid is attached to cysteine residues via thioester linkages. In palmitoylation the fatty acid is the 16-carbon palmitate, which exerts its effects mainly through interactions with lipid bilayers. Recently the F1-complex of the mitochondrial ATP synthase was identified as an interaction partner (Lyly et al., 2008). In peripheral cells, Ppt1 is routed to lysosomes via the mannose-6-phosphate pathway (Hellsten et al., 1996; Verkruyse and Hofmann, 1996). In neurons axonal and presynaptic localizations have been reported (Ahtiainen et al., 2003; Kim et al., 2008). The characteristic lysosomal accumulation of autofluorescent material occurs in most cell types in INCL, however the disease presents with neurological symptoms due to a selective loss of cortical and cerebellar neurons. Several proteins involved in anchorage, fusion and recycling events of SVs are palmitoylated (Huang and El-Husseini, 2005). In a recent report it was demonstrated that lack of Ppt1 activity causes abnormal sorting and membrane retention of several synaptic palmitoylated proteins critical to the recycling and regeneration of SVs. This leads to a marked reduction in the active pool of these organelles (Kim et al., 2008). The expression of Ppt1 is developmentally regulated in the mouse brain, but not in nonneural tissues (Suopanki et al., 1999).

Two mouse models of *Ppt1* deficiency exist, both generated by gene targeting. Gupta and colleagues (Gupta et al., 2001) generated a model in which a neomycin resistance cassette containing an in-frame stop codon replaces a portion of exon 9, resulting in premature termination of the Ppt1 polypeptide upstream of an essential catalytic amino acid. The brains of the affected mice showed granular osmiophilic deposits, and the Ppt1 activity of knockout brain extracts was dramatically lowered. However, the clinical course of the disease was milder than that observed in the human disease. At 50 days of age, half of the knockout mice displayed abnormal clasping behaviour, and at age 4-5 months the affected mice started showing signs of deterioration of motor function. Few mice survived beyond the age of 10 months. The other mouse model was generated by Jalanko and co-workers (Jalanko et al., 2005) by eliminating exon 4 through Cre/LoxP technology. Ppt1 activity was abolished in the knockout mice, and accumulation of autofluorescent material was observed in the brains. Phenotypically, a significant loss of vision was evident in the affected mice at age 14 weeks and seizures and clasping behaviour at 4 months. The average age of death was 6.5 months. Interestingly, *PPT1* has a homologue, *PPT2*, which

is not known to be mutated in humans. *Ppt2* knock-out mice display symptoms similar to those seen in the *Ppt1* deficient models (Gupta et al., 2001).

3.3. Congenital neuronal ceroid lipofuscinosis

Cathepsin D (CatD) is a lysosomal aspartic protease implicated in diverse biological processes, such as protein degradation, apoptosis and autophagy (Benes et al., 2008). It is synthesized in the rough ER as preprocathepsin D, which undergoes several modifications before taking its mature form, composed of heavy (34 kDa) and light (14 kDa) chains linked by non-covalent interactions (Benes et al., 2008). CatD is found intracellularly in most cell types in mammals. Like many other lysosomal hydrolases, CatD is targeted to lysosomes via the M6PR pathway. In addition, an M6PR independent mechanism of lysosomal targeting exists for CatD. This route remains poorly understood, but it has been suggested that the targeting is carried out by the interaction of pro-CatD with pro-saposin (Gopalakrishnan et al., 2004).

Lack of CatD function causes neuronal ceroid lipofuscinosis in humans (Steinfeld et al., 2006), American bulldogs (Awano et al., 2006), mice (Partanen et al., 2008) and sheep (Tyynela et al., 2000). In humans the designated abbreviation for the disease is CLN10. Neuropathologically the human disease is characterized by disruption of the normal architecture of the cerebral and cerebellar cortices, a marked loss of neurons and subcortical white matter, pronounced activation of microglia and astrocytes, and accumulation of lipofuscin in the surviving cells (Siintola et al., 2006). Similar to INCL, the storage deposits are not positive for subunit C of ATP synthase, but instead contain saposin D (Tyynela et al., 2000; Siintola et al., 2006). However, variable accumulation of subunit C of ATP synthase has also been reported in CatD deficient cells (Koike et al., 2000). The apparent discrepancy may be due to technical issues, such as antibody specifities. In humans, CatD deficiency leads to a congenital syndrome, presenting clinically with postnatal respiratory insufficiency, seizures and death within hours to weeks (Siintola et al., 2006). The findings in sheep are closely similar to those in the human disease (Tyynela et al., 2000).

Saftig and co-workers (Saftig et al., 1995) generated a mouse model of CatD deficiency, in which the open reading frame (ORF) of the *Ctsd* gene is interrupted in exon 4 and the truncated ORF only encodes the N-terminal quarter of mature CatD. In this mouse model, the disease phenotype is milder than in humans. The affected mice appear to develop normally for the first two weeks of life, then develop anorexia and seizures and die at postnatal day 26±1. During the last few days of life, the mice exhibit a decrease in spontaneous locomotion and an atactic gait (Saftig et al., 1995). In addition to neurological symptoms, the *Ctsd* deficient mice present with atrophy of the ileal mucosa which is followed in the terminal stage by intestinal necrosis and massive destruction of lymphoid cells in the spleen and thymus. These visceral symptoms are not observed in the human disease or in any of the other naturally occurring animal models of CatD deficiency. It remains unclear whether the visceral pathology reflects differences between

species, or if other unidentified molecular lesions occurred during the generation of the knockout mice.

Table 1 (following page): A summary of the diseases discussed in this work.

Disease	Defective gene	Inheritance	Sterol abnormality	Clinical picture	Selected reference(s)
Alzheimer's disease	Several affecting susceptibility to disease; APOE	Polygenic; risk gene dose-dependent for APOE	See chapter Implications for common neurodegenerative diseases	Progressive dementia	(Corder et al., 1993)
Atherosclerosis	Several affecting susceptibility to disease	Polygenic	Cholesterol-containing plaque formation in arterial walls; risk of disease associated with high plasma LDL and total cholesterol	Angina pectoris, claudication, stroke	(Stamler et al., 1986)
Cathepsin D deficiency	CTSD	Autosomal recessive	Accumulation of cholesteryl esters; altered levels of proteins related to cholesterol homeostasis	Postnatal respiratory insufficiency, seizures and death	(Steinfeld et al., 2006)
Desmosterolosis	DHCR24	Autosomal recessive	Perturbed cholesterol synthesis, elevated desmosterol levels	Severe congenital malformations	(FitzPatrick et al., 1998)
Familial hypercholesterolaemia	LDLR, PCSK9, APOB	Autosomal dominant	Decreased uptake of plasma LDL	Early onset atherosclerosis	(Blesa et al., 2008)
Huntington's disease	HTT	Autosomal dominant	Lowered cholesterol biosynthesis in neurons	Motor disturbances, cognitive disturbances, dementia	(Valenza et al., 2005)
Infantile neuronal ceroid lipofuscinosis	PPT1	Autosomal recessive	Enhanced synthesis of cholesterol in cortical neurons; altered expression of genes related to cholesterol homeostasis	Visual impairment, mental and motor retardation	(Haltia, 2006)
Niemann-Pick type C	NPC1 or NPC2	Autosomal recessive	Defective intracellular trafficking of cholesterol, accumulation of free cholesterol in late endosomes	Progressive neurodegeneration, hepatosplenomegaly, premature death	(Loftus et al., 1997; Naureckiene et al., 2000)
Smith-Lemli-Opitz syndrome	DHCR7	Autosomal recessive	Perturbed cholesterol synthesis, elevated 7-dehydrocholesterol levels	Congenital malformations, growth retardation, developmental delay	(Fitzky et al., 1998)
Tangier disease	ABCA1	Autosomal recessive	HDL deficiency, diminished ability to transport cholesterol out of cells	Neuropathy, hepatosplenomegaly, early onset atherosclerosis	(Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Rust et al., 1999)

4. Model systems to study CNS cholesterol

Studying cholesterol metabolism in the CNS is technically challenging. Although techniques to culture CNS cells *in vitro* have evolved remarkably over the years, several limitations still apply. Some CNS cells are considered to be post-mitotic (for example mature neurons and oligodendrocytes) (Raff et al., 1993). Primary CNS cells will survive and grow *in vitro* if prepared from the embryo or a neonate up to about one week old at the latest. The cells must then be allowed to mature and differentiate in culture, as the proliferating cell population mostly represents precursor cells (Herz et al., 1989). It is difficult to obtain cultures which purely represent one CNS cell type without any contaminating cells from other populations. Setting up pure cultures requires specialized techniques (Nagler et al., 2001). It should also be noted that the physiological function of CNS cells requires intimate contact between different cell types (Fields and Stevens-Graham, 2002), which is not easy to reconstitute in highly purified cultures. In addition, it may be simplistic to assume that even a relatively pure culture of primary CNS cells will represent only one cell type, as CNS cells form highly specialized subpopulations. Different subpopulations of CNS cells will also respond differentially to stimuli. As an example, cholesterol lowering affects neurite outgrowth differently in hippocampal and cortical neurons (Ko et al., 2005).

In order to support the growth and differentiation of CNS cells *in vitro*, the cell culture media must contain several types of nutrients and growth factors (Nagler et al., 2001). In the case of lipid research, this poses special problems: 10-20% serum is often used in the culture media to support CNS cells. The availability of serum lipoproteins is known to have effects on the cholesterol metabolism of cultured cells. However, the brain is protected by the BBB *in vivo*, and culture conditions high in serum are unphysiological for CNS cells. The cell culture media also often contain growth and signaling factors such as insulin as supplements, which may modulate cellular lipid metabolism (Vainio et al., 2006). Technically less demanding alternatives to primary cell cultures are immortalized cell lines. Spontaneuos generation of a human neuronal cell line has been reported (Ronnett et al., 1990), but spontaneous transformation is a rarity. Tumor cell lines, especially those arising from malignancies, are invariably dedifferentiated to varying degrees and their function may no longer represent that of primary CNS cells. Immortalized cell lines obtained through somatic cell fusion may provide models for the examination of specific neurochemical phenotypes (Wainwright et al., 1995). A promising emerging technique is the use of induced pluripontent stem cells, which can be differentiated *in vitro*. As these cells are obtained from differentiated cells through reprogramming by genetic manipulation or direct delivery of reprogramming proteins (Zhou et al., 2009) they provide new possibilities to study human disease in patient-specific cells.

As primary cultures of CNS cells can only be set up from embryonic or neonatal tissue, the mouse (*mus musculus*) has been the model organism of choice in most studies for a variety of reasons. Mice breed easily and they are small, relatively easy to maintain, genetically well characterized and a variety of techniques for genetic engineering exist. Most importantly, the mouse genome is relatively similar to human (Waterston et al.,

2002). Numerous mouse models for human diseases exist, generated both by random mutation (Loftus et al., 1997) and genetic engineering (Saftig et al., 1995; Jalanko et al., 2005). As an additional benefit, the inbred mouse strains which are used as disease models are genetically homogenous, and as such reduce the effects of heterozygosity on the phenotype.

The mouse is a good model for human physiology and pathology, but not a perfect one. There is considerable variation between the different inbred mouse strains, and species-specific differences between mice and men obviously exist at both DNA and protein level (Chang et al., 2001). Considering cholesterol metabolism for example, the composition of lipoprotein particles in the circulation of mice differs considerably from that in humans (Camus et al., 1983). The mouse is an "HDL animal," with high plasma HDL levels and low levels of LDL and VLDL, whereas humans have relatively high levels of plasma LDL and lower levels of HDL. Mice, unlike humans, are naturally deficient in cholesteryl ester transfer protein (CETP) (Barter et al., 2003), which is a plasma protein involved in the redistribution of CEs, TGs and phospholipids between lipoproteins. Differences also occur in the CNS. For example, in humans myelination begins in the second trimester and continues through the second postnatal year, whereas in mouse the process is postnatal (Dietschy, 2009). The phenotypes of the murine disease models may differ considerably from that seen in human diseases, regardless of whether the mouse models are generated through spontaneous mutation or gene technology. An example is the previously mentioned mouse model of cathepsin D deficiency, which has a milder neurological phenotype than humans, but presents with additional visceral symptoms.

Aims of the study

This study dealt with questions related to CNS cholesterol metabolism and its alterations in Niemann-Pick type C disease (NPC), infantile neuronal ceroid lipofuscinosis (INCL) and cathepsin D deficiency using mouse models. The aims of the three studies included in this thesis are listed below.

I) In the first study, we aimed to find out whether the synthesis and secretion of cholesterol in NPC1 deficient astrocytes were impaired. We also studied the particles in which astrocytes secrete cholesterol, and whether NPC2 is involved in this cholesterol efflux.

II) We investigated whether changes observed in the mRNAs of genes implicated in cholesterol biosynthesis are reflected as functional alterations in cholesterol biosynthesis in *Ppt1* deficient (INCL model) murine neurons.

III) We aimed to find out whether the structure and composition of myelin is alterd in cathepsin D deficient mice. As myelin is rich in lipids and especially cholesterol, we wanted to know whether cathepsin D dysfunction leads to dysregulation of cholesterol metabolism and altered lipid levels in the brains of cathepsin D deficient mice.

Materials and methods

The methods used in the original publications presented in this thesis are summarized in the table below. The roman numerals correspond to the original publications.

Method	Publication
Acute slice electrophysiological recordings	II
Analysis of cholesterol biosynthesis	I, II
Analysis of cholesterol efflux	I
Analysis of neuronal extensions	II
Analysis of polar lipids and fatty acyl and alkenyl chain composition	III
Culture of primary murine astrocytes	I
Culture of primary murine neurons	II
Electron microscopy	I, III
Flotation of membranes in a sucrose gradient	I
Glycosidase digestion	I
High performace liquid chromatography	I, II
Histochemistry, immunohistochemistry and microscopy	III
Immunocytochemistry and microscopy	I, II
Lowry protein determination	I, III
Measurement of cellular sterol amounts	I, II
Measurement of intracellular calcium levels	II
Neuronal stem cell proliferation and maturation	II
Proteomics	III
Radiolabeling of cells	I, II
Recombinant Semliki forest virus expression	I
SDS-PAGE and Western blotting	I, III
Size-exclusion choromatography	I
Thin layer chromatography	I, II, III
Transcript profiling	II

Experimental techniques personally used by the author are as follows: analysis of cholesterol biosynthesis and cholesterol efflux; culture of primary murine astrocytes; flotation of membranes in a sucrose gradient; high performance liquid chromatography; Lowry protein determination; immunocytochemistry and microscopy; measurement of cellular sterol amounts; radiolabeling of cells; recombinant Semliki forest virus expression; SDS-PAGE and Western blotting; thin layer chromatography.

Results and discussion

5. Secretion of sterols and the NPC2 protein from primary astrocytes

5.1. Astrocytes survive, differentiate and grow in low-serum culture conditions

BALB/c npc^{nih} mice heterozygous for an $Npc1$ disease mutation (Loftus et al., 1997) were obtained from the Penchev laboratory at the National Institutes of Health. Primary astrocyte cultures were prepared from striata from E16.5 $Npc1^{-/-}$, $Npc1^{+/-}$ and $Npc1^{+/+}$ mouse embryos, and cultured in low-lipoprotein conditions. The cells were seeded on poly-D-lysine coated dishes in Dulbecco's modified Eagle's medium supplemented with antibiotics, Hepes, L-glutamine and G5 supplement, which is designed for the growth and expression of astrocytes (Bottenstein, 1985). Fully serum-free cultures did not efficiently survive and expand, so the medium was supplemented with 1% characterized fetal bovine serum. Before any experiments were conducted, the cells were washed thoroughly (six times) with phosphate buffered saline (PBS). This is sufficient to remove traces of serum lipoproteins (Lafon-Cazal et al., 2003). The medium was then replaced with serum-free medium.

To confirm the identity of the cells, they were stained with antibodies for glial fibrillary acidic protein (GFAP), an intermediate filament expressed exclusively by astrocytes in the CNS (Eng et al., 1971; Budka, 1986). At least 85% of the cells were GFAP positive after 7-10 days in culture as determined by indirect immunofluorescence (I, Figure 1A). *In vivo*, GFAP-positive cells are a heterogenous population, with several clearly recognizable specialized subpopulations (for example Bergmann glia) and there is variability also within cultured cell populations. About 20% of hippocampal GFAP-positive astrocytes do not express ApoE even after exitotoxic injury (Xu et al 2006) and distinct GFAP-negative populations of astrocytes have been described (Walz and Lang, 1998). The 85% GFAP expression rate was deemed sufficient for the biochemical experiments presented in this work, as large cell populations were pooled for the experiments and GFAP-positive cells represented a clear majority. The 15% which did not stain for GFAP might be contaminating cells such as endothelial cells, or represent a GFAP-negative subpopulation of astrocytes. The presence of serum is known to promote GFAP expression in differentiating astrocytes (Raff et al., 1984). The low-serum culture conditions under which these experiments were conducted may contribute to the GFAP expression level.

Wild type and knockout cells were co-stained with filipin, a fluorescent antimicrobial agent which stains free cholesterol, and lysobisphosphatidic acid antibodies which stain the late endosomes (Kobayashi et al., 1999). The $Npc1$ deficient cells displayed perinuclear accumulations of filipin-positive material corresponding to the cholesterol-

filled late endosomes characteristic to NPC disease and co-localizing with staining for lysobisphosphatidic acid (I, Figure 1B, C).

5.2. Wild type astrocytes synthesize cholesterol but contain its precursors

Previous reports have suggested non-cholesterol sterols as the preferred product of the sterol synthesis pathway in CNS cells *in vitro* (Tabernero et al., 1993). To analyze whether this was true in primary cultures of CNS astrocytes, the cells were washed thoroughly with PBS and incubated with [^{14}C]cholesterol for 48 hours in order to allow the label to equilibriate with the pre-existing pools of cholesterol. The cells were then washed with PBS and labeled with [^{3}H]acetate in serum-free medium for 60 minutes, washed again and incubated for 2 or 18 hours in the presence of lovastatin and mevalonic acid. Lovastatin is used to block any further incorporation of the [^{3}H]acetate into the mevalonate pathway, and the medium is supplemented with some unlabeled mevalonic acid to promote cell survival in the absence of HMG-CoAR function (Marz et al., 2007). Acetate is utilized as a precursor for a variety of biomolecules including sterols.

To identify the [^{3}H] radioactivity incorporated in sterols, lipids were extracted according to the Bligh and Dyer method (Bligh and Dyer, 1959) and separated with high-performance thin layer chromatography (HP-TLC) followed by silver ion high performance liquid chromatography (Ag^{+}HPLC) used in tandem. Combined HP-TLC and HPLC was chosen because we wanted to resolve cholesterol as well as the major intermediates of sterol biosynthesis (Johnson et al., 1995). The results were corrected for protein amounts and [^{14}C]cholesterol, which was used as an internal standard to correct for extraction and analytical losses. After 2h chase, most [^{3}H] activity was not found as cholesterol, but its precursors zymosterol and desmosterol (I, Figure 2A). After 18h chase desmosterol was still a major [^{3}H] sterol in addition to cholesterol (I, Figure 2B). When the sterol content of unlabeled astrocytes was analyzed similarly with tandem HP-TLC and HPLC using UV detection, desmosterol was found to represent a major steady-state sterol in these cells (I, Figure 2D). The cholesterol:desmosterol ratio was 1.00:1.18 and other cholesterol precursors were present in trace amounts.

To determine whether desmosterol was indeed a component of membranes in these cells, we isolated an astrocyte membrane fraction by flotation in a sucrose gradient and extracted and analyzed sterols from each fraction by combined HP-TLC and Ag^{+}HPLC as described above. The cholesterol:desmosterol ratio in the membranes with equilibrium density between 0.8 and 1.7M was similar to that in total cell extract indicating that desmosterol is a major membrane sterol in these cells (Image G). To rule out the possibility that the accumulation of desmosterol was due to the *in vitro* culture conditions, we determined the relative amounts of unlabeled steady-state sterol species from freshly dissociated E16.5 striata. The cholesterol:desmosterol ratio was similar to that found in cultured astrocytes (data not shown).
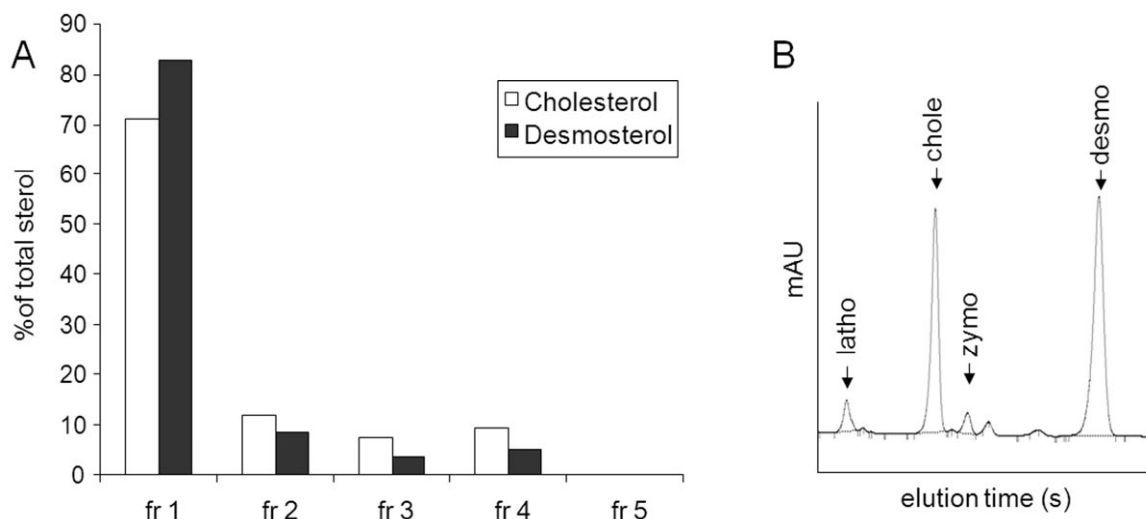
Image G: Flotation of wild-type astrocyte membranes in a sucrose gradient. Wild type astrocytes were collected, washed with PBS and suspended in 250mM sucrose, 10mM Hepes, 140mM KCl and protein inhibitors. A post-nuclear supernatant was prepared and the membranes flotated in a sucrose gradient. Five fractions were collected (1-5 top to bottom) and lipids extracted and analyzed by HP-TLC and HPLC using UV detection. Fraction 1 represents a fraction with an equilibrium density of 0.8-1.7M sucrose. A, the percentage of total cholesterol and desmosterol recovered in each fraction. B, HPLC analysis of fraction 1; latho, lathosterol; chole, cholesterol; zymo, zymosterol; desmo, desmosterol. Compare B with I, figure 2D.

5.3. Wild type astrocytes secrete sterols in heterogeneous particles

After 18h of chase, less [3H]sterol per mg protein was found in cells than after 2h chase (I, Figure 2C). Since there was no serum present to serve as acceptor for the radiolabeled sterols, the effluxed cholesterol was likely secreted in astrocyte-synthesized lipoprotein particles. According to our previous observations (Lusa et al., 2003), cholesterol precursors are efficiently effluxed to extracellular acceptors. We therefore analyzed whether the sterol intermediates were incorporated in the astrocyte-produced particles. Wild type astrocytes were again labeled with [3H]acetate and [14C]cholesterol as in chapter 5.2 to trace the appearance of newly-synthesized and steady-state sterols into serum-free culture medium, respectively. Cells and media were collected and lipids were extracted and analyzed by HP-TLC and Ag+HPLC. The amounts of [3H]sterols and [14C]cholesterol were found to increase in the culture medium as a function of time (I, Figure 3A) indicating sterol secretion by the astrocytes. Nearly half of [3H]sterols were still present as cholesterol precursors after 18h of chase (I, Figure 3A). To assess the size of the particles incorporating the effluxed sterols, medium from wild type radiolabeled labeled astrocytes was collected 18h after labeling, concentrated and subjected to size exclusion fractionation by gel filtration on a Superose 6HR column. Lipids were extracted from the fractions and analyzed by combined HP-TLC and HPLC analysis. Nearly all biosynthetic sterols and [14C]cholesterol were found in fractions 16–20 (I, Figure 3C, D). The material in this

fraction is larger than the 670kDa standard, suggesting that the particles are significantly larger than previously reported (LaDu et al., 1998).

To characterize the particles further, the sterol-containing fractions were concentrated and analyzed by negative-staining electron microscopy. This revealed heterogeneous spherical particles ranging in diameter from <15 nm to >30 nm, with an average diameter of 20 nm (I, Figure 4A). The electron microscopy results are in line with previously published characterizations of astrocyte-derived lipoprotein particles (LaDu et al., 1998; DeMattos et al., 2001). The size exclusion fractionation result was very reproducible in our hands, and may represent an artefact due to the processing of the samples or atypical migration of the particles by filtration.

5.4. The synthesis and efflux of newly synthesized sterols are not impaired in *Npc1*$^{+/-}$ or *Npc1*$^{-/-}$ astrocytes

The CNS relies on *de novo* cholesterol synthesis, and the majority of newly synthesized cholesterol in the mature CNS is thought to be derived from astrocytes and distributed to acceptor cells via lipoprotein particles (Mauch et al., 2001). Cholesterol accumulates in the late endosomes of NPC1 deficient CNS cells *in vivo* (Xie et al., 2000). In non-CNS NPC cells, the accumulating cholesterol is mostly LDL-derived. LDL particles like those found in the circulation are not present in the CNS, yet the most devastating symptoms of NPC target the brain. The mechanisms of cholesterol accumulation in the CNS in NPC1 deficiency may differ from those in the peripheral cells, as deletion of neither *apoE* nor *Ldlr* from the CNS affects the lifespan of *Npc1* deficient animals or prevent the accumulation of cholesterol in their brains (Xie et al., 2000). Sterol biosynthesis is known to be upregulated in peripheral NPC1 deficient cells. However, the total amount of cholesterol in the *NPC1*$^{-/-}$ brain does not appear to be increased (Vanier, 1999).

Even though subjects heterozygous for an NPC disease mutation do not develop clinical disease, abnormalities in cholesterol trafficking in cells from heterozygous individuals have been described. For example, cholesterol efflux to ApoAI has been found to be impared in cells heterozygous for an NPC1 disease mutation (Choi et al., 2003). Due to the differences between the regulation of peripheral and CNS sterol metabolism, we studied whether cholesterol synthesis and/or secretion were altered in *Npc1*$^{-/-}$ and *Npc1*$^{+/-}$ astrocytes.

Wild type, heterozygote and knockout astrocytes were labeled with [^{3}H]acetate and [^{14}C]cholesterol, and cells and media were collected after 18 hours of chase. The material was analyzed by tandem HP-TLC and Ag$^+$HPLC. No significant difference in the efflux of [^{3}H]sterols was observed between the genotypes (I, Figure 5B, D), albeit the rate of sterol biosynthesis was somewhat increased in heterozygous and knockout cells when compared to wild type (I, Figure 5A). No difference was seen in the relative amounts of precursor sterols (I, Figure 5A, 5C; compare with 2D). In conclusion, *Npc1* deficient and heterozygous astrocytes displayed a mildly increased rate of sterol biosynthesis when compared to wild type cells, but the difference was not significant. No significant difference in the secretion of sterols was observed between the genotypes.

Cholesterol precursors differ in function and biophysical behaviour from cholesterol. They are not retained in the ER where most enzymes involved in cholesterol biosynthesis are located, but are transported to the plasma membrane together with cholesterol (Johnson et al., 1995). They affect membrane fluidity and functions of membrane proteins (Vainio et al., 2006), and they have been implicated in physiological processes in some tissues. In the male reproductive tissues, desmosterol has been suggested to influence the motility of spermatozoa flagellae (Connor et al., 1998), and specific cholesterol precursors have also been implicated in the activation of meiosis in oocytes and spermatocytes (Byskov et al., 1995; Rozman et al., 2005). It is interesting that these cells also have a high percentage of polyunsaturated fatty acids in their membrane lipids (Connor et al., 1998), a characteristic also observed in astrocytes (Moore, 2001). Whether cholesterol precursors have specific functions in the CNS remains an open question.

We found that desmosterol is a major membrane sterol in embryonic murine astrocytes *in vitro*, and incorporated into astrocyte-derived *de novo* lipoprotein particles. Desmosterol is not a substrate for CYP46A1, which converts CNS cholesterol into 24S-hydroxycholesterol, the oxysterol considered to be responsible for a significant part of sterol efflux from the CNS (Ohyama et al., 2006). CYP46A1 is almost exclusively localized in CNS neurons in the normal brain with little expression in glial cells (Ohyama et al., 2006). One of the possible advantages of desmosterol accumulation during development of the mammalian brain is that desmosterol is not available for CYP46A1 for conversion into cerebrosterol. Desmosterol may thus be efficiently secreted for neurons by astrocytes and avoid conversion into cerebrosterol and thereby excretion from the CNS. A recent study on highly pure cultures of CNS cells shows significant differences in the post-squalene cholesterol precursor profiles of astrocytes and neurons (Nieweg et al., 2009). Additionally, neurons were found to produce sterols inefficiently and have a low capacity to up-regulate the synthesis compared to astrocytes, lending support to the idea of neurons relying at least in part on astrocyte-derived sterols.

Some of the effects of cholesterol precursors may be conveyed via their different effects on gene transcription or modulation of raft-mediated processes. Different sterols may not be able to promote the formation of rafts with the same efficiency as cholesterol (Xu et al., 2001; Vainio et al., 2006), or support raft-dependent biological functions (Vainio et al., 2006). Desmosterol has been found to function as an LXR ligand and upregulate the expression of ABCA1 in an enantiomer-dependent manner independently of the SREBP pathway, whereas 7-dehydrocholesterol and cholesterol were found to have no LXR-mediated effect (Yang et al., 2006). Whether the accumulation of desmosterol during CNS development is linked to the other, non-enzymatic roles of DHCR24, remains to be addressed.

In this study, lathosterol was the most efficiently effluxed sterol with cholesterol and desmosterol being effluxed at comparable rates for both labeled (I, Figure 3B) and unlabeled sterols (I, Figure 4B). Previously, desmosterol in particular has been shown to be avidly effluxed to extracellular acceptors in several cell types (Johnson et al., 1995). The differences in efflux between the various sterols may be due to their differential trafficking in the cell. For instance, the availability of newly synthesized lathosterol to methyl-β-cyclodextrin on the plasma membrane could be inhibited by pharmacological

polymerization of actin, but not by the Golgi-disrupting agent brefeldin A (BFA) (Lusa et al., 2003), indicating a transport mechanism independent of Golgi function but requiring an intact cytoskeleton. In contrast, the availability of newly synthesized cholesterol to cyclodextrin was in part BFA sensitive (Lusa et al., 2003).

5.5. The synthesis and secretion of NPC2 are not impaired in *Npc1*$^{-/-}$ astrocytes

Lack of NPC2 function produces a clinical phenotype indistinguishable from NPC1 deficiency (Naureckiene et al., 2000), and the two NPC proteins have been suggested to function in tandem (Naureckiene et al., 2000; Infante et al., 2008). NPC2 binds cholesterol at submicromolar affinity (Ko et al., 2003) and it can be secreted (Kirchhoff et al., 1996). In cells cultured from NPC1 patients, NPC2 has been shown to be mistrafficked (Blom et al., 2003). It was of interest to investigate whether NPC2 is secreted normally from NPC1 deficient astrocytes. Human *NPC2* cDNA was expressed in murine astrocytes using the Semliki Forest virus (SFV) expression system, and analyzed by Western blotting. Endoglycosidase H and N-glycosidase F (PNGase F) digestions revealed differential glycosylation patterns between the murine and human NPC2 proteins (I, Figure 6A). This was utilized to differentiate between the endogenous and overexpressed proteins.

Both endogenous and recombinant NPC2 were found to be secreted into the medium in both *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ astrocytes (I, Figure 6C), and quantitation of the Western blots demonstrated moderately increased levels of endogenous NPC2 in *Npc1*$^{-/-}$ as compared with *Npc1*$^{+/+}$ cells (I, Figure 6B, C). The enhanced expression was reflected in an enhanced secretion of the endogenous protein (I, Figure 6B). The secretion of the recombinant protein was more efficient in both genotypes than the secretion of the endogenous protein. No difference in the secretion of the recombinant NPC2 protein was found between the *Npc1* genotypes (I, Figure 6C). The efficient secretion of overexpressed NPC2 may be due to the saturation of the M6PR system due to the high amount of overexpressed protein. In conclusion, the synthesis and secretion of NPC2 are not inhibited in primary astrocytes lacking functional NPC1.

5.6. Secretory NPC2 protein is not associated with the bulk of secreted cholesterol in astrocytes

To investigate if NPC2 was co-secreted with the cholesterol containing particles or if large amounts of secreted, cholesterol-bound NPC2 might explain part of the sterol efflux from the astrocytes, we equilibrium-labeled wild type astrocytes with [^{3}H] cholesterol. They were then infected with recombinant SFVs to overexpress wild type NPC2, incubated overnight in the absence of radiolabel, and the medium was then collected. The collected medium was subjected to size-exclusion fractionation, and the fractions were analyzed by liquid scintillation counting and immunoblotting with anti-NPC2 antibodies. The majority of the [^{3}H]cholesterol radioactivity was found in fractions 16-20 (I, Figure 7A), whereas

the NPC2 signal was detected in fractions 36-40, which corresponds to the expected NPC2 monomer size (I, Figure 7B). The overexpression of NPC2 did not produce changes in the fractions in which [^{3}H]cholesterol appeared (I, Figure 7A). Based on these results, it cannot be ruled out that NPC2 is secreted in its cholesterol-bound form, as the used method may not be sensitive enough to detect labeled cholesterol secreted in an equimolar ratio with NPC2. However, the majority of cholesterol secretion from these cells was found to be independent of NPC2 secretion.

Our findings that *Npc1* deficient astrocytes are capable of synthesizing cholesterol and secreting sterols in nascent lipoprotein particles as efficiently as wild type astrocytes have since been confirmed by others (Karten et al., 2005). In *Npc1* deficient mice *in vivo*, it is possible that lipoproteins are being produced normally by astrocytes, but that the target cells are not able to metabolize the lipoprotein particles in a normal manner. This might lead to the accumulation of free cholesterol in the late endosomes and subsequent neurodegeneration. Recently, Zhang and coworkers (Zhang et al., 2008) showed that *Npc1* expression under the *Gfap* promoter in *Npc1* deficient mice led to increased survival, decreased neuronal lipid load, restoration of neurofilament proteins and fertility, and alleviation of neurodegeneration. In these mice, NPC1 function is only restored in astrocytes, not in cells thought to be recipients of astrocyte-derived lipoproteins. The alleviated neurodegeneration of these mice implies that the pathogenetic mechanism is dependent on astrocyte function, but the exact mechanisms require further study. It remains possible that the aberrant metabolism and accumulation of lipoprotein-derived cholesterol is secondary to unidentified pathogenetic mechanism(s) underlying the disease.

6. Cholesterol homeostasis is disturbed in cultured cortical *Ppt1*$^{\Delta ex4}$ neurons

6.1. Altered expression of enzymes involved in lipid metabolism in *Ppt1*$^{\Delta ex4}$ neurons

The starting point for investigating cholesterol balance in *Ppt1*$^{\Delta ex4}$ neurons was that our collaborators performed comparative gene expression analysis of cultured cortical wild type and *Ppt1*$^{\Delta ex4}$ neurons. Global gene expression studies of approximately 14000 mouse genes showed significant changes in the expression of 135 genes. According to the Gene Ontology classifications, one of the affected biological processes was lipid metabolism, more specifically sterol metabolism. Several genes encoding for enzymes of the mevalonate pathway of cholesterol biosynthesis showed increased expression. Specifically, these were HMG-CoA synthase, HMG-CoA reductase and 3β-hydroxysterol Δ(14)-reductase (converts Δ8,14,24-dimethylsterol and Δ8,14-dimethylsterol to Δ8,24-dimethylsterol and Δ8-dimethylsterol, respectively) (Table 2). Interestingly, in some experimental setups HMG-CoA synthase and not HMG-CoA reductase functions as the rate-limiting enzyme in the mevalonate pathway in glia (Volpe and Obert, 1981). In

addition, the isoprenoid metabolism components farnesyl diphosphate synthase and isopentenyl diphosphate isomerase were upregulated (Table 2).

Table 2. Significantly upregulated genes encoding for enzymes of the mevalonate pathway in cultured Ppt1$^{\Delta ex4}$ mouse embryonic cortical neurons.

Gene symbol	Gene name	Fold change	Function
Hmgcs1	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1	3.10-2.14	Mevalonate pathway
Idi1	Isopentenyl-diphosphate Δ isomerase 1	2.82	Isoprenoid synthesis
Tm7sf2	3β-hydroxysterol Δ(14)-reductase	2.82	Cholesterol biosynthesis
Fdps	Farnesyl diphosphate synthetase	2.75	Isoprenoid synthesis
Hmgcr	3-Hydroxy-3-methylglutaryl-coenzyme A reductase	1.89	Mevalonate pathway

6.2. Cholesterol biosynthesis is increased in *Ppt1*$^{\Delta ex4}$ neurons

To study whether the changes in expression profiles led to changes in function, we cultured cortical neurons from wild type and *Ppt1*$^{\Delta ex4}$ cortices to study whether their ability to synthesize cholesterol was altered. The cells were cultured *in vitro* for 12 days in DMEM supplemented with antibiotics, 0,5mM glutamine, 2,5mM glutamic acid, 10mM Hepes pH 7.4 and B27 supplement. The cells were washed with PBS and incubated with [^{3}H]acetate in serum-free medium for 60 minutes. After another PBS wash, the cells were incubated for 20-22 hours in the presence of lovastatin and mevalonate. Unlabeled mevalonate was added because inhibition of HMG-CoAR activity without supplementing mevalonate promotes apoptosis in neurons (Marz et al., 2007).

As predicted by the expression data, sterol biosynthesis was found to be upregulated in the knock-out neurons. The synthesis of the end-product cholesterol was upregulated the most, to ~2,5-fold that observed in wild type cells (II, Figure 4A). A significant effect was also seen in the synthesis of desmosterol, the penultimate precursor of cholesterol on the Bloch pathway, but not lathosterol, a late intermediate in the Kandutsch-Russell pathway (II, Figure 4A). When the total sterol amounts were analyzed, the amount of cholesterol was found to be unchanged in the knock-out neurons as compared to wild type, whereas the amounts of desmosterol and lathosterol were found to be reduced (II, Figure 4B). A reduction was also observed in the amount of the early cholesterol precursor, lanosterol (II, Figure 4B).

Ppt1 has been shown to be able to remove palmitic acid from S-acylated proteins *in vitro* (Camp and Hofmann, 1993). Palmitoylation can target proteins to lipid rafts (Herincs et al., 2005) and Ppt1 deficiency has been reported to lead to altered lipid composition of rafts (Goswami et al., 2005). Increased sterol synthesis we observed in *Ppt1* deficient neurons may reflect compensatory mechanisms to preserve raft functions. *Ppt1* deficient neurons display a reduction in the active pool of synaptic vesicles (Kim et al., 2008). Cholesterol is known to participate in the formation, transport and function of these organelles (Thiele et al., 2000; Klopfenstein et al., 2002). Thus, one explanation for the observed upregulation of sterol synthesis would be to maintain adequate amounts of cholesterol for synaptic functions.

The relative lack of precursor sterols in INCL neurons may reflect their increased conversion to cholesterol. The loss of sterol was not experimentally investigated in this study, but it is of interest to note that no significant changes were observed in known LXR target genes. In addition to genes encoding for enzymes involved in the biosynthesis of cholesterol, a gene encoding for the sterol-binding protein Stard4 was found to be upregulated in *Ppt1* deficient neurons in our study. The exact function of the sterol-binding Stard4 has not been pinned down, but it has been implicated in intracellular cholesterol transport (Rodriguez-Agudo et al., 2008). The upregulation of this gene in *Ppt1* deficient neurons may be linked to the altered cholesterol homeostasis observed in these cells.

Low levels of lanosterol observed in the $Ppt1^{\Delta ex4}$ neurons are in line with the upregulation of the gene encoding for 3β-hydroxysterol Δ(14)-reductase, an enzyme downstream of lanosterol in the cholesterol biosynthetic pathway. Lanosterol, the first intermediate in the cholesterol biosynthetic pathway with a sterol backbone, has been suggested to promote the degradation of HMG-CoAR in an Insig-dependent manner (Song et al., 2005). Lanosterol also does not bind efficiently to the sterol-sensing domain of SCAP to suppress SREBP-mediated regulated transcription of SRE-bearing genes (Radhakrishnan et al., 2004). Thus, it seems logical that lanosterol be promptly converted to later intermediates when continuing efficient cholesterol synthesis is desired.

7. Cathepsin D deficiency leads to dysmyelination and altered levels of neutral lipids

Mutations in the gene encoding for the lysosomal protease CatD underlie the severe neurological disorder congenital neuronal ceroid lipofuscinosis. The affected infants have atrophic brains and diminished myelin already at birth (Fritchie et al., 2009). The mouse model of CatD deficiency presents with milder symptoms developing later in life (Saftig et al., 1995). However, recent studies have uncovered pathological changes indicative of a loss of white matter (Haapanen et al., 2007) and disruption of CNS axonal myelin sheaths (Partanen et al., 2008) in CatD deficient mice. Disruption of CatD function has been reported to disrupt the expression of proteins central to cholesterol homeostasis, such as ABCA1 (Haidar et al., 2006). As most of CNS sterol resides in myelin, we hypothesized that the sterol composition of the brains of terminal $Ctsd^{-/-}$ mice would be altered.

7.1. Accumulation of cholesteryl esters and decrease in triglycerides in *Ctsd* deficient brain tissue

Although CatD deficiency has been shown to perturb the expression of genes involved in maintaining cholesterol homeostasis (Haidar et al., 2006), previous investigators found no difference in total cholesterol amounts between wild type and *Ctsd* knockout mouse brains (Jabs et al., 2008). To investigate the sterol balance in the knockout tissue further, we analyzed the amounts of neutral lipids, including CE, in the brains of wild type and *Ctsd* deficient mice with HP-TLC analysis at the preterminal age of P24. The amounts of CE were found to be significantly increased in the knockout tissue, whereas the amounts of triglycerides (TG) were significantly decreased. The increase in CE was 5-6 fold compared to wild type, and TG was found to be decreased circa 50-60% (III, Figure 3A, B). Cholesterol accumulates intracellularly in CatD deficient cells (Haidar et al., 2006), however we found no difference in the level of free cholesterol calculated per total protein in *Ctsd* deficient versus wild type tissue (data not shown), in line with previous observations (Jabs et al., 2008).

7.2. Amounts of proteins associated with cholesterol metabolism and myelin are altered in *Ctsd* deficient brain

The pattern of protein expression in synaptosomes from *Ctsd* knockout mice was analyzed by quantitative shotgun proteomics and three essential myelin proteins were found to be significantly reduced in the affected mice: proteolipid protein (PLP), myelin basic protein (MBP) and 2', 3'-Cyclic Nucleotide 3' Phosphodiesterase (III, Table 1). The protein amounts of the cholesterol carriers ApoE and ApoAI were found to be increased 2-fold in the knockout synaptosomes (III, Table 1). Next the structure of myelin in the brains of *Ctsd* knockout mice was investigated by immunohistochemistry. Proteolipid protein staining revealed a remarkable loss of this essential myelin component (Boison and Stoffel, 1994) in the hippocampi, anterior commisura and corpora callosa of knockout mice as compared to control (III, Figure 1A, 1B). In the thalami of knockout mice PLP staining was present in clusters instead of the fine fibrous structures typical to wild type mice (III, Figure 1B). The myelinated structures in *Ctsd* knockout brains also displayed decreased staining with the dye Luxol fast blue, which is commonly used to visualize myelin structures (III, Figure 1B). However, no clear difference in MBP staining could be observed (III, Figure 1B).

To further investigate the effect of CatD deficiency on proteins involved in cholesterol homeostasis, we examined the amounts of ApoAI, ApoE and ABCA1 by semi-quantitative Western blotting. ABCA1 expression (Haidar et al., 2006) and ApoE processing (Zhou et al., 2006) have previously been linked to CatD function. Contrary to the proteomics data (III, Table 1), no changes were found in the protein amounts of ApoAI (III, Figure 5C). In accordance with previous reports (Haidar et al., 2006), the protein amounts of ABCA1 were found to be drastically reduced in *Ctsd* deficient tissue (III,

Figure 5B). As also supported by the proteomics data presented in this publication, the amount of ApoE was found to be increased circa 3-fold (III, Figure 5A).

7.3. Abnormal lipid composition and structure of myelin in *Ctsd* deficient brain

Myelin is extremely rich in lipids with a distinctive lipid profile (Martinez, 1982). Therefore, the profile of polar lipids and fatty acyl and alkenyl chain composition in *Ctsd* knockout brains was analyzed. Plasmalogen-derived alkenyl chains and 20- and 24-carbon saturated and monounsaturated fatty acids typical for glycosphingolipids were found to be significantly reduced, but polyunsaturated species were significantly increased in the knockout brains (III, Figure 3). Myelin is rich in plasmalogens and glycosphingolipids (Siegel et al., 1999), so the observed decrease of about a third of these lipids suggests a corresponding loss of myelin in the knockout mice. When the main molecular species of PE plasmalogens were analyzed, a significant increase in the polyunsaturated species was observed at the expense of monounsaturated ones (III, Figure 3). The differences were most pronounced in ethanolamine plasmalogens. In human white matter, over 50% of ethanolamine plasmalogens are composed of monounsaturated acyl chains, whereas polyunsaturated species dominate in the gray matter (Han et al., 2001). The observed reduction in monounsaturated species at the expence of polyunsaturated ones in *Ctsd*^{-/-} mice may reflect a decrease in white matter.

To further dissect whether the altered myelin protein composition was due to an aberrant assembly of myelin or dysmyelination, ultrastructural analysis by electron microscopy was performed. The axonal myelin sheaths appeared thinner in *Ctsd* knockout brains compared to control (III, Figure 2A-D). The ratio of axon diameter to myelin thickness, a tightly regulated parameter in the healthy brain (Fraher and O'Sullivan, 2000), was increased in *Ctsd* knockout brain. Additionally, some axons in the *Ctsd*^{-/-} brains were found to be enveloped in an abnormally thick myelin sheath, suggesting abnormal assembly of myelin structures (III, Figure 2E).

Myelin consists of cholesterol-enriched membranes (Saher et al., 2005), and a breakdown of these membranes may lead to an excess of free cholesterol in the CNS. To avoid toxicity, excess cholesterol may be stored by cells as CE, packaged into lipoprotein particles for excretion or, in the CNS, converted to oxysterols for elimination. The observed accumulation of CE in the *Ctsd* deficient brains may reflect increased incorporation of cholesterol to CE to prevent toxicity. The decreased amount of TG in the *Ctsd*^{-/-} brain could be due to increased degradation of TG and the incorporation of their fatty acids into the accumulating CE.

Additionally, the lipoprotein-mediated route of cholesterol transport is fundamentally disrupted in the CNS of these mice. In peripheral cells, ABCA1 facilitates the lipidation of lipid-free ApoAI or lipid-poor HDL particles, and it has been shown to have a role in lipidating astrocyte-derived lipoprotein particles in the CNS (Wahrle et al., 2004). The expression of ABCA1 has been suggested to regulate the amount of ApoE, as ApoE has been found to be significantly decreased in the CNS of ABCA1 knockout mice (Wahrle et

al., 2004). CatD has been reported to contribute to the proteolysis of both lipidated and unlipidated ApoE (Zhou et al., 2006). Together with the decreased ABCA1 amount, the increased ApoE protein amount may point to a failure of the $Ctsd^{-/-}$ tissue to efficiently degrade ApoE particles. These particles are likely to be poorly lipidated due to the paucity of ABCA1.

Intrestingly, ApoAI levels were found to be dramatically increased in the CNS of genetically engineered mice with a brain-spesific ABCA1 knockout (Karasinska et al., 2009), suggesting that ABCA1 might play a role in regulating ApoAI levels in the brain. ApoAI expression has not been unequivocally demonstrated in the CNS parenchyma, but it is highly expressed in primary porcine brain capillary endothelial cells (Panzenboeck et al., 2002). ApoAI appears to influence CNS sterol metabolism through mechanisms not yet precisely identified, as decreased sterol amounts have been reported in the cortices of adult ApoAI knockout mice compared to control (Fagan et al., 2004). SR-BI (Karasinska et al., 2009) and LDLR (Dehouck et al., 1997) are both expressed in brain capillary endothelial cells. In fact, LDLR expressed at the BBB has been utilized in the selective transfer of proteins to the CNS by fusing the LDLR-binding domain of apolipoproteins to the target proteins (Spencer and Verma, 2007). This strongly suggests a possibility for selective cholesterol transport through the BBB. In porcine brain capillary endothelial cells, ABCA1 is localized to the basolateral membrane and apoAI is secreted in this direction, consistent with the generation of lipoprotein particles in the brain parenchymal fluid (Panzenboeck et al., 2002). Considering the dramatically decreased expression of ABCA1 in *Ctsd* knockout mice, it is likely that the mechanisms governing cholesterol transport events at the BBB are also disturbed in these animals.

Implications for common neurodegenerative diseases

This thesis consists of studies which focus on disruption of cholesterol metabolism in neurodegenerative diseases. The approach is based on rare, recessively inherited monogenic diseases for which mouse models exist. Rare diseases such as those investigated in this work can potentially provide clues to the pathobiology of more common and complex diseases. One of the most intensely studied diseases in this context is Alzheimer's disease (AD), the most common cause of dementia worldwide.

At the tissue level, AD is characterized by synapse and neuron loss, senile plaques and neurofibrillary degeneration in vulnerable areas of the brain. The accumulation of amyloid peptide (Aβ) is a characteristic of the senile plaques (Masters et al., 1985) and hypothesized to be central to AD pathogenesis (Siegel et al., 1999). The neurofibrillary degeneration is characterized by the intraneuronal accumulation of an abnormal, hyperphosphorylated form of the microtubule-associated protein tau (Grundke-Iqbal et al., 1986).

Accumulating evidence points to a potential role for cholesterol in the pathogenesis of AD. Genetic studies in the early nineties revealed a strong, dose-dependent association between late-onset AD incidence and the *APOE* genotype ε4 (Corder et al., 1993). Single nucleotide polymorphisms in the gene encoding for ApoJ, *CLU*, were recently linked to AD in a large genome-wide association study (Harold et al., 2009). Early epidemiological studies reported that the prevalence of AD was lower in patients taking statins (Jick et al., 2000; Wolozin et al., 2000), but recent clinical studies have brought these findings into question (Rea et al., 2005; Arvanitakis et al., 2008). Some studies have suggested that a high total serum cholesterol level in midlife is a risk factor for AD (Kivipelto et al., 2001), but others have found no such association (Slooter et al., 1999).

γ-secretase, the activity of which is required for the release of Aβ from its precursor amyloid precursor protein (APP), cleaves its substrates within the plane of the membrane in a cholesterol-sensitive process called regulated intramembrane proteolysis (Iwatsubo, 2004). The cleavage activity of BACE1 is required at the β-cleavage site of APP to initiate its amyloidogenic processing, and this process is thought to be raft-dependent (Ehehalt et al., 2003). ACAT inhibitors have been shown to modulate Aβ levels and plaque formation *in vivo* in mouse models (Hutter-Paier et al., 2004), and animal studies have suggested that a high-cholesterol diet can increase Aβ levels (Refolo et al., 2000). Additionally, knocking down ACAT1 in cultured neuroglioma cells leads to a significant decrease in Aβ generation (Huttunen et al., 2007). Statins have been found to alter Aβ production: atorvastatin increases the activity of the non-amyloidogenic α-secretase pathway in neuroblastoma cells (Parvathy et al., 2004). Modulating cholesterol content in cultured hippocampal neurons affects secretory and intracellular Aβ levels (Fassbender et al., 2001).

Several similarities exist in the pathological features between AD and the rarer neurodegenerative disorders presented in this work. Neurofibrillary tangles immunologically identical to those found in AD have been described in the brains of human NPC patients (Distl et al., 2003), raising the possibility of shared mechanisms of neuronal degeneration in these two diseases. Hyperphosphorylation dissociates tau from

microtubules, with which it normally associates. The dissociated tau then self-aggregates into paired helical filaments, which have the ability to form larger aggregates, *id est* neurofibrillary tangles. The mechanisms of neurodegeneration mediated by pathologically aggregated tau remain poorly defined. They may be related to toxic gain-of-function of the hyperphosphorylated tau, or to loss-of-function of the physiological tau via its diminished association with microtubules and impact on transport processes (Ballatore et al., 2007). It is of worth to note that tau pathology is sufficient on its own to drive neurodegeneration (Tanemura et al., 2002). In addition to the presence of neurofibrillary tangles in NPC brain, NPC1 deficient cultured cells and mouse brains have been reported to accumulate Aβ (Yamazaki et al., 2001). Wild type cells treated with the class 2 amphiphile U18666A, which produces an NPC-like phenotype, also accumulate the fibrillogenic Aβ42 in late endosomes (Yamazaki et al., 2001).

A possible point of convergence for the pathogenetic mechanisms in neurodegenerative disorders linked to cholesterol is the endosomal system, which is essential in the intracellular processing and trafficking of cholesterol. The NPC1, Ppt1 and CatD proteins themselves localize to the endo-lysosomal organelles. Moreover, abnormalities of the endosomal and/or lysosomal compartments have been described in NPC, CatD deficiency and INCL. In AD neurons endosomes are abnormally large and the endocytic pathway has been reported to be activated, as determined by elevated levels of Rab4 immunoreactive protein and increased translocation of rabaptin 5 to endosomes (Cataldo et al., 2000). In this study, Rab4 was used as a marker for recycling and rabaptin 5 as a marker for internalization. Enlargement of Rab5-positive early endosomes is an early indicator of cell pathology in AD (Nixon, 2004). Similar changes can be observed in cells overexpressing Rab5, which also increases the production of Aβ (Grbovic et al., 2003).

The deficient activity of lysosomal proteins may lead to altered generation or clearance of Aβ. CatD is not required for the baseline amyloidogenic processing of APP (Saftig et al., 1996), but it may have a role in the clearance of Aβ peptides (Hamazaki, 1996). It has been suggested to have β-secretase activity when highly expressed (Mathews et al., 2002). A variant of Ppt1 was recently identified as influencing the ectodomain shedding of APP (Schobel et al., 2006). Deficiency of a functional NPC1 has been demonstrated to lead to increased levels of Aβ (Yamazaki et al., 2001) and an enhanced localization of presenilin in endosomes (Burns et al., 2003), suggesting that the defects present in NPC may lead to abnormal amyloidogenic processing of APP.

The levels of lysosomal CatD are increased in neurons in AD vulnerable regions even before the onset of major pathology (Cataldo et al., 1991), and in both NPC and AD, CatD has been shown to be redistributed to early endosomes (Jin et al., 2004). It has been suggested that increased CatD levels in AD result from Aβ-induced regulation of CatD levels (Hoffman et al., 1998; Jin et al., 2004) or from accelerated endocytic uptake and delivery of cargo to the endocytic system (Cataldo et al., 2008). CatD has been suggested to participate in the proteolysis of several proteins and peptides central to AD: tau (Kenessey et al., 1997), ApoE (Zhou et al., 2006) and Aβ (Sakamoto et al., 2006). We found ApoE protein amount to be elevated in preterminal *Ctsd*^{-/-} mouse brain (III), in line with decreased ApoE proteolysis by CatD. ApoE influences the association of astrocytes

with amyloid deposits and it is essential in astrocyte-mediated clearance of Aβ (Koistinaho et al., 2004). ApoE interaction with Aβ is isoform-specific and depends on the lipidation status of ApoE (Tokuda et al., 2000), which in turn is dependent on ABCA1 function (Hirsch-Reinshagen et al., 2004). Although *Ctsd* deficient CNS tissue has increased ApoE compared to wild type (III), it may be poorly lipidated due to the paucity of ABCA1. This may affect Aβ clearance and thus provide a mechanistic link between CatD deficiency and AD.

Enhanced CatD immunostaining has been demonstrated in NPC1 deficient brains in areas exhibiting both accumulation of intracellular cholesterol and neurodegeneration (Liao et al., 2007), and ApoE has been shown to be elevated in NPC1 deficient brain tissue (Karten et al., 2005). No impairment was seen in the secretion of *de novo* sterol into acceptor-free medium from $Npc1^{-/-}$ murine astrocytes (I). As ApoE is the main apolipoprotein secreted by astrocytes, it seems likely that ApoE secreted from these cells is lipidated. The increased amounts of ApoE and CatD observed in NPC1 deficient cells and tissue may reflect compensatory effects to clear material accumulating intracellularly in NPC1 deficiency, including but not necessarily limited to Aβ. Recently, another intriguing molecular link between NPC and AD was uncovered when Winkler and coworkers reported that NPC1 and M6PR copurify with active human PS1 (Winkler et al., 2009). This points to a possible interaction between NPC-associated proteins and the proteolysis of APP, as PS1 functions as a part of the γ-secretase complex active in amyloidogenic processing of APP (Wolfe et al., 1999). Overexpression of cation-dependent M6PR has previously been demonstrated to mistarget CatD to early endosomes and to increase Aβ secretion (Mathews et al., 2002). At present, there is not enough information available to determine whether the possible interactions between NPC1, M6PR and PS1 influence this.

In (II), we found the biosynthesis of cholesterol and its precursor desmosterol to be enhanced in *Ppt1* deficient cortical neurons, while the actual amounts of lathosterol, desmosterol and lanosterol were decreased. DHCR24, the enzyme responsible for the conversion of desmosterol to cholesterol is also known as Seladin-1 (short for SELective Alzheimer's Disease INdicator 1), as it has been shown to be selectively down-regulated in large pyramidal neurons in vulnerable brain areas in Alzheimer's disease (Greeve et al., 2000). It has been shown to confer prosurvival effects through several, not yet fully defined routes. The activity of *CYP51*, which encodes for lanosterol 14α-demethylase, the enzyme immediately downstream of lanosterol on the mevalonate pathway, is regulated at the transcriptional level through SCAP/SREBP (Rozman et al., 1996). The *CYP51* promoter also contains a cAMP regulatory element involved in immediate early gene response (Fink et al., 2005). Alterations in the amounts of lanosterol and other pre-cholesterol sterols observed in neurodegenerative conditions may represent a complex response to inflammatory as well as sterol-synthesis specific cues. LXR function has been implicated in inflammation (Bensinger and Tontonoz, 2008), and provides another point of convergence between neurodegenerative processes and control of cholesterol homeostasis. LXRs have been suggested to influence the development of neuropathology in AD (Zelcer et al., 2007) and NPC1 (Repa et al., 2007) and modulate the inflammatory response of glia (Zelcer et al., 2007).

Conclusions

Cholesterol is a ubiquitous component of all mammalian cells and vital to life. In diseases leading to profound disturbances of cell homeostasis, impairment of central cell functions such as processing of cholesterol is to be expected.

I) Murine astrocytes synthesize cholesterol and retain cholesterol precursors *in vitro*. Synthesis of sterols and assembly and secretion of sterol-carrying lipoprotein particles are not inhibited in $Npc1^{-/-}$ or $Npc1^{+/-}$ astrocytes. The majority of sterol is secreted independently of secreted NPC2 both in wild type and $Npc1^{-/-}$ astrocytes.

II) Several genes encoding for enzymes in the mevalonate pathway were found to be upregulated in $Ppt1^{\Delta ex4}$ neurons. The changes in gene expression were reflected as changes in sterol homeostasis: cholesterol biosynthesis was upregulated in $Ppt1^{\Delta ex4}$ neurons and the amounts of cholesterol precursors were decreased.

III) $Ctsd^{-/-}$ mouse brains display altered myelin architecture and have decreased amounts of lipids which are characteristically found in myelin. $Ctsd^{-/-}$ brains accumulate cholesteryl esters, but have lowered amounts of triglycerides as compared to wild type. The changes in the lipid profile are accompanied by altered levels of proteins involved in lipoprotein-mediated cholesterol transport, including ABCA1 and ApoE.

In light of accumulating evidence, it seems likely that disturbances in CNS cholesterol balance play a specific role in the pathology of several neurodegenerative diseases. The studies presented in this work elucidate the function of astrocytes in a model of NPC1 disease and provide evidence of disturbances of cholesterol homeostasis in mouse models of INCL and cathepsin D deficiency. Identifying and characterizing cholesterol defects in these disease models may eventually contribute towards the development of therapeutic interventions. To resolve the mechanisms of disease, more information about the processes governing cholesterol homeostasis in the healthy CNS is needed.

Acknowledgements

Mrs. Glossbrenner, thank you for the gift of English. My high school teachers are thanked for their patience and understanding. Without you, my academic career would have been very short indeed.

My PBL group at Medical school is thanked for covering my back most of the time when I was late from school during those hectic years. Petri is especially thanked for his friendship and for allowing the Cardiology teacher to keep thinking I had a respectable job at McDonald's.

To my other friends: thank you for being there, and thank you for reminding me that Medicine may not be the only thing in life.

I wish to thank my parents Kari and Ulla for their limitless belief in my abilities and for all of their love and support through the years. My brother Sampo, thank you for our debates and making me consider new angles.

Kimmo, thank you for your unwaivering, even-headed and pragmatic support. You are truly the rock upon which I build, and I feel privileged to love you. Pi the dog and Rho the puppy are thanked for reminding me to live in the moment. The best thing in life is to love and be loved in return, and I'm so fortunate to have that.

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