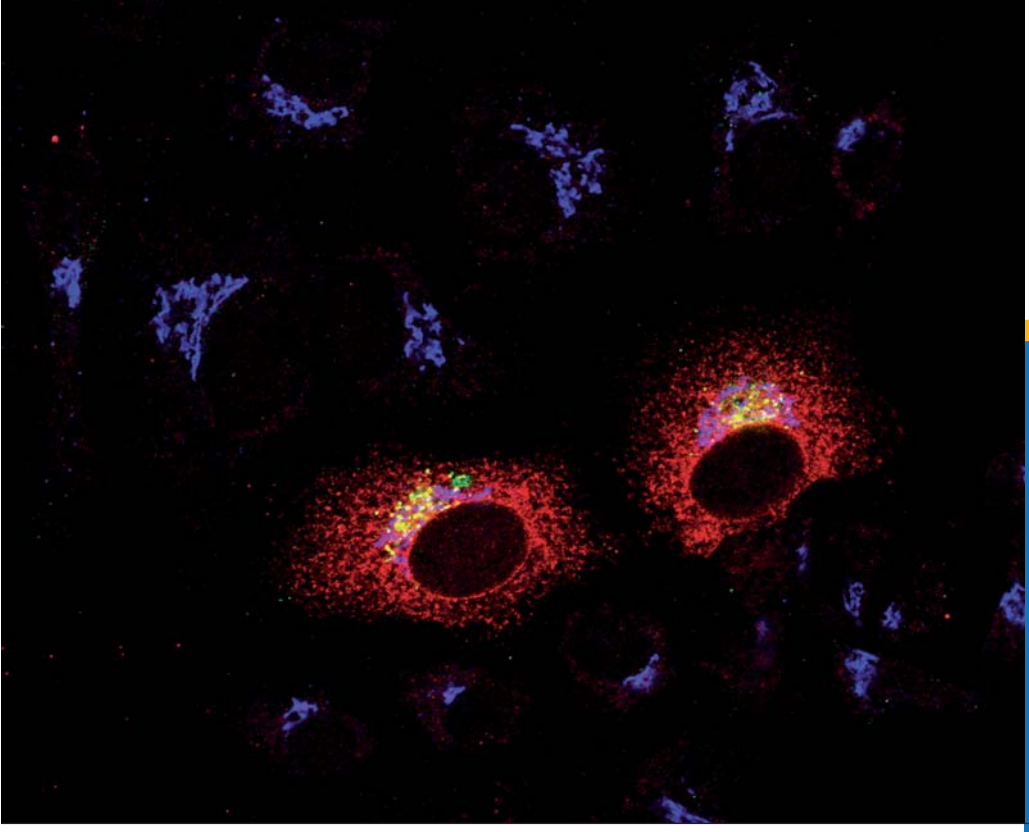

Annina Lyly

Molecular Interactions Underlying Neuronal Ceroid Lipofuscinoses CLN1 and CLN5

Publications of the National Public Health Institute A 17/2008

Department of Molecular Medicine
National Public Health Institute
and
Faculty of Medicine, University of Helsinki, Finland

Helsinki, Finland 2008

Annina Lyly

MOLECULAR INTERACTIONS UNDERLYING NEURONAL CEROID LIPOFUSCINOSES CLN1 AND CLN5

A C A D E M I C D I S S E R T A T I O N

To be presented with the permission of the Medical Faculty of the University of Helsinki, for public examination in the Niilo Hallman lecture hall, Hospital for Children and Adolescents, Helsinki University Central Hospital, on June 6th , 2008, at 12 noon.

National Public Health Institute, Helsinki, Finland

and

Faculty of Medicine, University of Helsinki, Finland

Helsinki 2008

Publications of the National Public Health Institute
KTL A17 / 2008

Julkaisija-Utgivare-Publisher

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ISBN 978-951-740-821-9
ISSN 0359-3584
ISBN 978-951-740-822-6 (pdf)
ISSN 1458-6290 (pdf)

Kannen kuva - cover graphic: Annina Lyly

Yliopistopaino
Helsinki 2008

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To my family

Annina Lyly, Molecular interactions underlying neuronal ceroid lipofuscinoses CLN1 and CLN5
Publications of the National Public Health Institute, A17/2008, 98 Pages
ISBN 978-951-740-821-9; 978-951-740-822-6 (pdf-version)
ISSN 0359-3584; 1458-6290 (pdf-version)
http://www.ktl.fi/portal/4043

ABSTRACT

NCL disorders, neuronal ceroid lipofuscinoses, comprise the most common group of neurodegenerative diseases in children. The incidence of these hereditary disorders is approximately 1 : 12 500 worldwide. Features common to all NCLs include accumulation of the autofluorescent intracellular storage material, ceroid lipofuscine, in the tissues of patients, and progressive neuronal death, leading to blindness, psychomotor retardation, and premature death. Disease-causing mutations have been characterized in eight different genes so far, but the cellular mechanisms leading to neurodegeneration are still largely unknown.

Infantile NCL (INCL), together with congenital NCL, is the most severe form of the NCL disorders. The clinical manifestations include progressive visual failure, epileptic seizures, and ataxia. INCL patients die approximately at the age of ten. The *CLN1* gene encoding for the PPT1 (palmitoyl protein thioesterase 1) enzyme is mutated in INCL. PPT1 removes a palmitate moiety from proteins in experimental conditions, but its substrates *in vivo* are not known. CLN5 is a Finnish variant of late infantile NCL ($vLINCL_{Fin}$). The defective gene, *CLN5,* encoding for the CLN5 protein, was found in 1998. The first symptoms of $vLINCL_{Fin}$, visual failure and motor deterioration, are observed before school age. Progressive mental retardation leads to death at early adulthood. The function of CLN5 has remained unknown.

The aim of this thesis was to elucidate the disease mechanisms of these two NCL diseases enriched in Finland. The focus has been on the molecular interactions of the defective proteins. It was previously known that the intracellular localization of PPT1 is different in neurons than in other cell types. In neurons, it localizes mainly to axonal presynaptic compartments instead of lysosomes, where it is found in other cell types. In this study we observed that the processing of PPT1 was different between neuronal cells and connective tissue cells, fibroblasts. Furthermore, the intracellular trafficking of overexpressed PPT1 was found to differ from the classic trafficking pathway utilized by another lysosomal enzyme, AGA. These findings implicate novel properties for the PPT1 enzyme in neurons, and are significant when designing therapeutic strategies for INCL. PPT1 was also found in high molecular weight complexes and its activity was separated into two fractions. The protein that

formed a complex with PPT1 and purified with it was identified as the β-subunit of the mitochondrial ATP synthase. Later we found that PPT1 interacted with the entire F_1-complex that includes the β-subunit. The F_1-complex has been localized to the cell surface in addition to mitochondria, and in hepatocytes it has been shown to participate in HDL metabolism. The connection between PPT1 and the localization of F_1-subunits was studied utilizing the INCL-disease model, the genetically modified Ppt1-deficient mice. Our results show that in Ppt1-deficient neurons, the amount of F_1-subunits was specifically increased on the cell surface, while this was not detected in fibroblasts. Studies conducted with neuronal cells and serum samples further showed several changes in lipid metabolism both at the cellular and systemic levels, since the cellular uptake of apolipoproteins and the lipoprotein particle size were altered in Ppt1-deficient mice compared to controls. Thus, the identification of the first interaction partner for PPT1 led on to the studies connecting the altered neuronal function and disturbancies in lipid metabolism in INCL.

In the last part of the thesis, the focus was to elucidate the interactions between different NCL proteins. The reasoning behind this was the possible common metabolic route affected in NCL disorders, leading to similar clinical and neuropathological manifestations. CLN5 was chosen as the center molecule, as it was already known to interact with two other NCL proteins, CLN2 and CLN3, and it represents an NCL protein with an entirely elusive function. We detected three novel interactions between CLN5 and other NCL proteins, including an interaction with PPT1. The interaction between CLN5 and PPT1 turned out to be especially strong, as both proteins affected the intracellular transport of each other. In addition, we detected an interaction between CLN5 and the F_1-complex, further supporting a functional connection between CLN5 and PPT1. The multiple interactions between CLN5 and other NCL proteins suggest a connection between the NCL subtypes at the cellular level, where they possibly modify the phenotypes of each other. This is supported by the bidirectional interconnection of CLN5 and PPT1 in intracellular protein sorting.

The main results of this thesis elicit information about the neuronal function of PPT1. The connection between INCL and neuronal lipid metabolism is strengthened by the characterization of the first interaction partner for PPT1, introducing a new perspective to this rather poorly characterized subject. The evidence of the interactions between NCL proteins provides the basis for future research trying to untangle the NCL disease mechanisms and the development of therapies for these diseases.

Keywords: neurodegeneration, developmental disturbance, neuronal ceroid lipofuscinosis, interaction partner, lipid metabolism, metabolic route.

Annina Lyly, Molecular interactions underlying neuronal ceroid lipofuscinoses CLN1 and CLN5
Kansanterveyslaitoksen julkaisuja, A17/2008, 98 sivua
ISBN 978-951-740-821-9; 978-951-740-822-6 (pdf-versio)
ISSN 0359-3584; 1458-6290 (pdf-versio)
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TIIVISTELMÄ

Neuronaaliset seroidilipofuskinoosit eli NCL-taudit ovat yleisin lasten hermostoa rappeuttavien sairauksien ryhmä. Näiden perinnöllisten tautien esiintymistiheys on maailmanlaajuisesti noin 1 : 12 500. Kaikille NCL-taudeille yhteinen piirre on solunsisäinen autofluoresoiva kertymämateriaali, ”seroidi lipofuskiini”, sekä etenevä hermosolujen tuhoutuminen, joka johtaa sokeutumiseen, psykomotorisen kehityksen häiriintymiseen ja ennenaikaiseen kuolemaan. NCL-tauteja aiheuttavia mutaatiota on tähän mennessä löydetty kahdeksasta eri geenistä, mutta hermosolukuolemaan johtavat solutason tapahtumat ovat suurelta osin tuntemattomia.

Infantiili NCL (INCL) on synnynnäisen NCL:n ohella NCL-taudeista vakavin. Taudin kliiniseen kuvaan kuuluu mm. sokeutuminen, epilepsia ja ataksia. INCL-potilaat kuolevat noin kymmenvuotiaina. INCL-taudissa palmityyliproteiini-tioesteraasia eli PPT1:tä koodittava *CLN1*-geeni on mutatoitunut. PPT1:n on todettu poistavan palmitaatti-rasvahappotähdettä proteiineista koeolosuhteissa, mutta sen toiminta elimistössä on vielä tuntematon. CLN5 on suomalainen muunnos myöhäisestä lapsuusiän NCL-taudista ($vLINCL_{Fin}$). Taudissa vioittunutta CLN5-proteiinia koodittava *CLN5*-geeni löydettiin vuonna 1998. Taudin ensimmäiset oireet, näön heikkeneminen ja motorisen kehityksen taantuminen, havaitaan kouluikään mennessä. Etenevä kehitysvammaisuus johtaa potilaan kuolemaan varhaisessa aikuisiässä. CLN5-proteiinin toimintaa ei toistaiseksi tunneta.

Tässä väitöskirjatyössä pyrittiin selvittämään näiden kahden Suomessa rikastuneen NCL-taudin tautimekanismeja keskittymällä taudeissa vioittuneiden proteiinien molekyylitason vuorovaikutusten selvittämiseen. Aiemmin oli tiedossa, että PPT1:n solutason paikantuminen on erilaista hermosoluissa verrattuna muihin solutyyppeihin. Hermosoluissa PPT1 rikastuu niiden viejähaarakkeisiin, aksoneihin, lysosomien sijaan. Väitöskirjatyössä havaittiin, että PPT1:n prosessointi on erilaista hermosolujen ja sidekudossolujen välillä. PPT1:n solunsisäisen kuljetuksen havaittiin eroavan toisen, klassista kuljetusreittiä käyttävän lysosomaalisen AGA-entsyymin kuljetuksesta. Löydöksemme osoittavat, että PPT1:llä on hermosoluille erityisiä ominaisuuksia, jotka voivat olla merkityksellisiä INCL:n hoitomuotoja kehitettäessä. PPT1:n todettiin muodostavan suurikokoisia komplekseja, ja olevan

aktiivinen kahdessa eri muodossa. PPT1:n kanssa kompleksin muodostava proteiini tunnistettiin mitokondriaaliseksi ATP-syntaasin β-alayksiköksi. Tutkimuksissamme selvisi, että PPT1 on vuorovaikutuksessa ATP-syntaasin F_1-kompleksin kanssa, jonka osa β-alayksikkö on. F_1-kompleksin tiedetään paikantuvan mitokondrioiden lisäksi solun pinnalle, ja osallistuvan maksasoluissa HDL-rasva-aineenvaihduntaan. F_1-alayksiköiden solutason paikantumista tutkittiin INCL-tautimallin, Ppt1-siirtogeenisten hiiren hermosoluissa ja sidekudossoluissa. Havaitsimme, että F_1-alayksiköiden määrä on lisääntynyt hermosolun pinnalla INCL-soluissa, kun taas sidekudossoluissa muutosta ei havaittu. Jatkotutkimukset hermoston soluilla ja veriseeruminäytteillä osoittivat rasva-aineenvaihdunnan muutoksia sekä solutasolla että systeemisesti, sillä apolipoproteiinien soluunkuljetuksessa ja lipoproteiini-partikkeleiden koossa havaittiin merkittäviä eroja Ppt1-siirtogeenisten ja kontrollihiirten välillä. PPT1:n ensimmäisen vuorovaikutuskumppanin tunnistaminen pohjusti täten uudenlaisen yhteyden löytymistä hermosolujen muuttuneen toiminnan ja rasva-aineenvaihdunnan välillä INCL-taudissa.

Väitöskirjan viimeisessä osatyössä keskityttiin NCL-proteiinien välisten vuorovaikutusten selvittämiseen, sillä NCL-tautien yhtenäisen kliinisen kuvan takana ollaan arveltu olevan NCL-proteiinien toimiminen saman aineenvaihduntareitin varrella. CLN5-proteiini valittiin keskusproteiiniksi, sillä sen tiedettiin vuorovaikuttavan jo kahden muun NCL-proteiinin, CLN2:n ja CLN3:n kanssa. Havaitsimme näiden lisäksi kolme uutta NCL-proteiinien välistä sitoutumista. CLN5:n ja PPT1:n välinen vuorovaikutus osoittautui erityisen voimakkaaksi, sillä se vaikutti proteiinien paikantumiseen solussa. Lisäksi havaitsimme, että myös CLN5 sitoutuu F_1-kompleksiin, edelleen vahvistaen mahdollista toiminnallista yhteyttä PPT1:n ja CLN5:n välillä. CLN5:n vuorovaikutukset lukuisten muiden NCL-proteiinien kanssa viittaavat solutason yhteyden olemassaoloon eri NCL-tautien välillä, vaikuttaen mahdollisesti tautien ilmiasuun. Tätä tukee löydös, jossa osoitettiin molemminpuolinen vaikutus CLN5- ja PPT1-proteiineille toistensa solunsisäisessä kuljetuksessa.

Väitöskirjan tulokset tuovat lisätietoa PPT1:n toiminnasta hermosolussa. PPT1:n ensimmäisen vuorovaikutuskumppanin kuvaus vahvistaa INCL-taudin ja PPT1:n yhteyttä hermosolun rasva-aineenvaihduntaan tuoden mielenkiintoisen näkökulman aiheeseen, josta toistaiseksi tiedetään varsin vähän. Lisäksi todisteet NCL-proteiinien välisistä vuorovaikutuksista solutasolla vahvistuivat, antaen pohjaa tuleville tutkimuksille NCL-tautimekanismien selvittämiseksi ja hoitomuotojen kehittämiseksi.

Asiasanat: hermosolukuolema, kehityshäiriö, neuronaalinen seroidilipofuskinoosi, vuorovaikutuskumppani, rasva-aineenvaihdunta, aineenvaihduntareitti.

CONTENTS

ABBREVIATIONS

2BP	2-bromopalmitate
aa	amino acid(s)
ANCL	adult neuronal ceroid lipofuscinosis (CLN4)
apoA-I	apolipoprotein A-I
apoE	apolipoprotein E
ATP	adenosine triphosphate
BMP	bis(monoacylglycero)phosphate
bp	base pair
cDNA	complementary DNA
CHO cells	Chinese hamster ovary cells
CLN	ceroid lipofuscinosis, neuronal (in gene and protein nomenclature)
CLN5/Cln5	human/mouse CLN5 gene
CLN5/Cln5	human/mouse CLN5 protein
CNS	central nervous system
COS-1 cells	African green monkey kidney cells
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
ER	endoplasmic reticulum
GTP	guanosine triphosphate
HDL	high-density lipoprotein
HeLa cells	human cervical tumour cells
INCL	infantile neuronal ceroid lipofuscinosis (CLN1)
JNCL	juvenile neuronal ceroid lipofuscinosis (CLN3)
kb	kilobase(s)

kDa	kilodalton(s)
LBPA	lysobisphosphatic acid
MPR	mannose 6-phosphate receptor
mRNA	messenger RNA
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC12 cells	rat adrenal medulla pheochromocytoma cells
PCR	polymerase chain reaction
PPT1/Ppt1	human/mouse palmitoyl protein thioesterase 1 protein (CLN1)
PTM	post-translational modification
RNA	ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulphate
SFV	Semliki Forest virus
TIRF	total internal reflection fluorescence
UTR	untranslated region
vLINCL	variant form of late infantile neuronal ceroid lipofuscinosis (CLN2)
$vLINCL_{Fin}$	Finnish variant form of late infantile neuronal ceroid lipofuscinosis (CLN5)
wt	wild type

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, referred to in the text by their Roman numerals:

I **Lyly Annina**, von Schantz Carina, Salonen Tarja, Kopra Outi, Saarela Jani, Jauhiainen Matti, Kyttälä Aija, Jalanko Anu. Glycosylation, transport, and complex formation of palmitoyl protein thioesterase 1 (PPT1) – distinct characteristics in neurons. *BMC Cell Biology,* 2007 Jun 12;8:2

II **Lyly Annina**, Marjavaara Sanna, Kyttälä Aija, Uusi-Rauva Kristiina, Luiro Kaisu, Kopra Outi, Martinez Laurent, Tanhuanpää Kimmo, Kalkkinen Nisse, Suomalainen Anu, Jauhiainen Matti, Jalanko Anu. Deficiency of the INCL protein Ppt1 (palmitoyl protein thioesterase 1) results in changes in ectopic F_1-ATP synthase and altered cholesterol metabolism. *Human Molecular Genetics,* 2008 May 15;17(10):1406-1417.

III **Lyly Annina***, von Schantz Carina*, Heine Claudia, Schmiedt Mia-Lisa, Sipilä Tessa, Jalanko Anu, Kyttälä Aija. CLN5 interacts with multiple NCL proteins and is connected to PPT1/CLN1 via intracellular transport and F_1-ATPase interaction. Submitted.

* These authors contributed equally to this work.

1 INTRODUCTION

Disorders resulting from degenerative changes in the nervous system are progressive and incurable. They become more common as the population ages and influence the lives of millions of people worldwide. Cellular events leading to neurodegeneration are still mostly unknown. Environmental and inherited factors affect neuron survival, and neurodegenerative diseases are often the sum of both factors. Monogenic diseases, besides being important study subjects in their own right, offer a model for studying the mechanisms of neurodegeneration. Neuronal ceroid lipofuscinoses, or NCLs, are a group of monogenic, autosomal recessively inherited diseases affecting mostly children. NCLs cause severe neurodegeneration, resulting in the deterioration of motor and mental skills and leading to premature death (Haltia 2003). NCLs resemble each other histopathologically; the accumulation of storage material in most cell types, especially in neurons, is associated with massive and selective neuronal loss. In Finland, four forms of NCL are more common than elsewhere, namely infantile (INCL), Finnish variant late infantile ($vLINCL_{Fin}$), juvenile (JNCL), and Northern epilepsy. Here, the focus will be on INCL (CLN1) and $vLINCL_{Fin}$ (CLN5).

The incidence of INCL in Finland is 1:20000, meaning that approximately three affected children are born each year. The first symptoms appear around the age of one, when a child starts to lose already learnt cognitive and motor skills. As the disease progresses, irritability, visual failure, progressive decline in psychomotor skills, and epileptic seizures appear. By the age of three, the patients have lost active movements and social and visual contact with their environment, and they die around the age of ten (Santavuori et al. 2000). $vLINCL_{Fin}$ is a rare form of NCL, but in Southern Ostrobothnia its local incidence is 1:1500 (Jarvela 1991). Altogether, about 30 CLN5 cases have been reported. Slight clumsiness and learning problems are the first symptoms of CLN5 and they appear before school age. Ataxia, myoclonia, and epilepsy start before the age of 10 and the patients become wheelchair-bound at 9-13 years of age (Santavuori et al. 2000).

INCL is caused by mutations in the *CLN1* gene that disrupt the structure of the encoded protein, palmitoyl protein thioesterase 1 (PPT1) (Vesa et al. 1995). PPT1 is a soluble enzyme cleaving a palmitate moiety from proteins. Its *in vivo* substrates are still unknown, as is the reason for neuron-specific cell loss. Finnish variant LINCL patients carry mutations in the *CLN5* gene, preventing the yet unknown function of the corresponding CLN5 protein (Savukoski et al. 1998). In this thesis, the aim was to explore the interaction partners for PPT1 and CLN5 in order to gain insight into their normal functions and to the mechanisms behind the neuronal death in this group of diseases.

2 REVIEW OF THE LITERATURE

2.1 PROTEIN PALMITOYLATION

2.1.1 Dynamic modification for membrane association

Proteins are the functional derivatives of the encoding genes. In a protein, the linear data stored in a gene is transformed into a three-dimensional, operational unit that affects other proteins and genes via interactions with them. It has been estimated that the expression of the 20 000 to 25 000 protein-encoding genes in the human genome results in more than one million protein products (Nørregaard Jensen 2004). The complexity of the human proteome is mainly the consequence of alternative splicing and post-translational modifications (PTM). PTMs are covalent processing events on amino acids of the polypeptide backbone that change the properties of a protein. Over 300 PTMs have been described to occur physiologically, of which the most common are glycosylation, phosphorylation, glycosylphosphatidylinositol (GPI) anchor formation, proteolytic cleavage, ubiquination, and fatty acid modifications such as palmitoylation (Garavelli 2004).

Palmitate, a 16-carbon saturated fatty acid, is commonly found in plants and animals. It is the major fatty acid in palm oil, and thus the name (Fedeli and Jacini 1971). When attached to proteins, palmitate regulates their functions in diverse ways. Palmitoylation facilitates the membrane-association of proteins and regulates protein stability. Palmitate is not only a membrane anchor for proteins, as transmembrane proteins are also palmitoylated, for instance for lipid raft association (Linder and Deschenes 2007). Protein palmitoylation can occur in cysteine residues in two ways, N- and S-palmitoylation. The first refers to the addition of palmitate to the N-terminal cysteine residue, resulting in a spontaneous conversion of thioester linkage to a stable amide linkage present in secreted signalling proteins such as Sonic hedgehog (Miura and Treisman 2006). In most cases, palmitate is attached to a protein through a reversible, covalent thioester linkage in a process called S-palmitoylation (**Figure 1**). Palmitate turnover can be constitutive or regulated, depending on the protein (Linder and Deschenes 2007). Protein palmitoylation has been a difficult subject to study due to its dynamic nature and lack of a specific palmitoylation consensus sequence in the potential palmitoylprotein. Palmitoylation has traditionally been studied by *in vivo* [^{3}H]-palmitate labelling and detection of radiolabelled palmitate in the protein of interest. New proteomic methods based on acyl-biotinyl exchange chemistry have provided the opportunity for large-scale

identification of palmitoylated proteins at the whole-organism level (Drisdel and Green 2004, Roth et al. 2006).

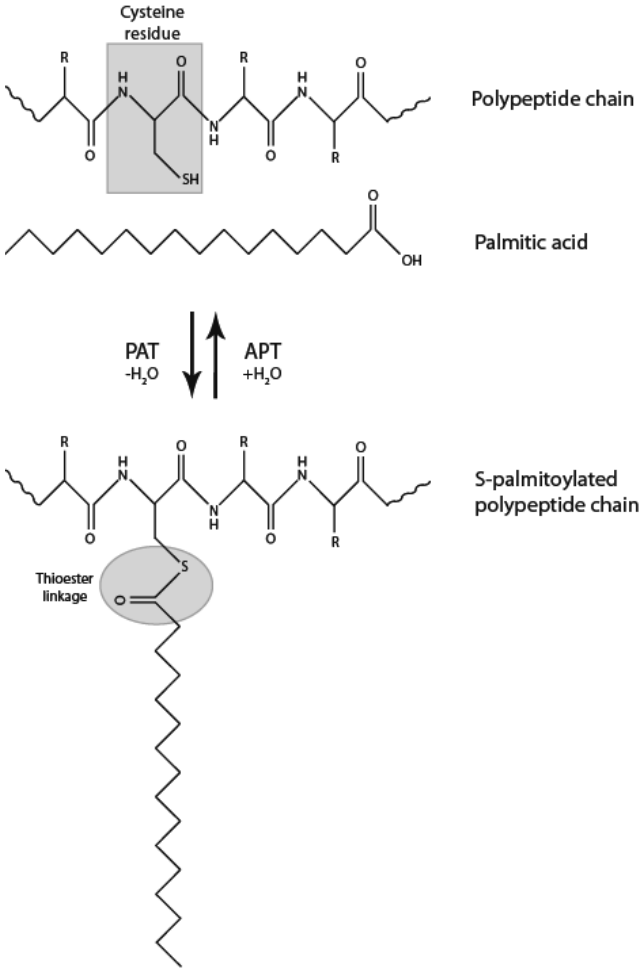

Figure 1. ***Protein S-palmitoylation occurs at a cysteine residue in a polypeptide chain via a reversible thioester linkage. Protein acyl transferases (PAT) catalyze palmitoylation, and acyl protein thioesterases (APT) catalyze the hydrolysis of palmitoyl protein.***

2.1.2 Palmitoylation in the cytoplasm

S-palmitoylation can occur at the cytoplasmic face of a membrane or in the lumen of the secretory pathway (**Figure 2**). Palmitoylation of cytoplasmic proteins is mostly mediated by the recently discovered protein acyltransferases (PATs), transmembrane enzymes that were first identified in yeast (Lobo et al. 2002, Roth et al. 2002) and later in mammals (Fukata et al. 2004). PATs possess a 50-residue cysteine-rich domain with a conserved Asp-His-His-Cys (DHHC) motif needed for the PAT acivity. DHHC proteins are located both in endomembranes (e.g. ER and Golgi) and the plasma membrane, and function to palmitoylate cytosolic proteins. Palmitoylation can occur in tandem with other, stable lipid modifications such as prenylation or myristoylation (Smotrys and Linder 2004). Ras proteins and heterotrimeric G-proteins (guanyl nucleotide-binding proteins) are small GTPases and examples of soluble, dually lipidated proteins. Farnesylation of H- and N-Ras provides only a weak membrane-affinity, and palmitoylation increases their membrane association (Hancock et al. 1990). Palmitoylation also defines their subcellular localization, driving Ras proteins to the plasma membrane (Rocks et al. 2005). Additionally, the α-subunits of G-proteins need palmitoylation for plasma membrane targeting and binding with βγ subunits. Before the G-protein-coupled receptor activation, Gα is bound to βγ and protected from depalmitoylation. When the receptor is activated, Gα dissociates from βγ subunits and activates adenylyl cyclase. Palmitoylation enhances this activation, and depalmitoylation of Gα is needed for the regulation of downstream effects (Resh 2006). Many transmembrane proteins are palmitoylated by the DHHC proteins in the cytoplasmic cysteines near the transmembrane domain. For example, many SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins, involved in vesicular fusion, possess such palmitoylation (Roth et al. 2006).

2.1.3 Intralumenal palmitoylation

In the secretory pathway, palmitoylation of secreted signalling proteins is regulated by enzymes belonging to a diverse family of membrane-bound O-acyltransferases (MBOAT) (Hofmann 2000). Many genes of this family (e.g. Rasp, Skinny Hedgehog, and Sightless) have been reported to be involved in the Hedgehog signalling pathway through N-palmitoylation (Bijlsma et al. 2004). S-palmitoylation has been demonstrated to be important for Wnt-protein signalling (Komekado et al. 2007, Kurayoshi et al. 2007). Wnt signalling plays a key role during development; however, it is also involved in various adult-onset diseases such as cancer, osteoporosis, Alzheimer's disease, and schizophrenia (Caricasole et al. 2005, Patel and Karsenty 2002). Wnt binds its receptors Frizzled and a low-density lipoprotein

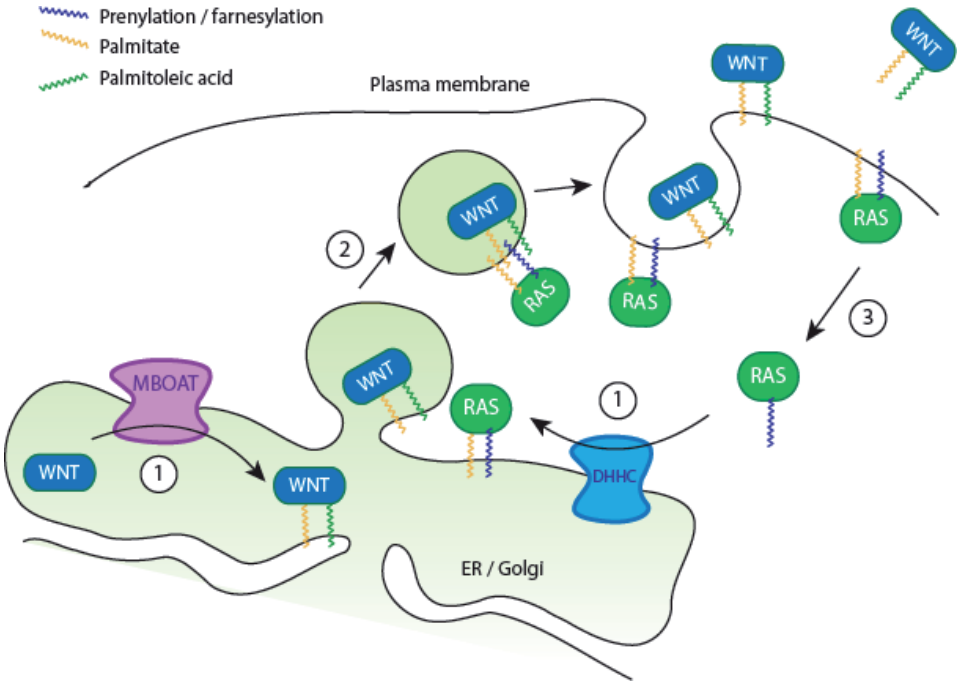

Figure 2. ***Schematic representation of the intracellular sites of protein palmitoylation. 1) Palmitoylation of Wnt occurs intralumenally and is catalyzed by proteins belonging to the MBOAT-family. Palmitoylation of Ras, catalyzed by the DHHC-proteins, occurs at the cytosolic face of the ER or Golgi membrane. 2) Palmitoylated proteins traffic through the secretory pathway to the plasma membrane or become secreted. 3) Depalmitoylated Ras is released from the plasma membrane and is able to diffuse to endomembranes to become repalmitoylated. Modified from*** (Hausmann et al. 2007, Linder and Deschenes 2007, Resh 2006)

related protein (LRP) on the cell surface and activates numerous signalling pathways, which are usually divided into two main classes, the canonical and noncanonical pathways. The canonical pathway results in stabilization of the transcription factor β-catenin and activation of Wnt target genes (Cadigan 2002). The noncanonical pathways include activation of several second messengers such as Rho GTPases, calcium, and heterotrimeric G-proteins (Veeman et al. 2003). Wnt possesses lipid modifications in two conserved cysteine residues, Cys77 and Cys209. The first cysteine is palmitoylated, and the second residue obtains an unsaturated lipid moiety, palmitoleic acid (Takada et al. 2006, Willert et al. 2003).

Porcupine, a putative acyltransferase, has been suggested to catalyse both acylations of Wnt. Palmitoylation on Cys77 is required for Wnt-3a to bind and activate its receptors (e.g. LRP6), either by increasing the affinity between the proteins or by targeting Wnt to the lipid raft microdomains (Komekado et al. 2007). Palmitoleic acid modification is required for the correct intracellular targeting and secretion of Wnt (Takada et al. 2006).

Interestingly, soluble palmitoylproteins that function primarily inside the organelles of the secretory pathway rather than become secreted have not been reported so far. One explanation for this might be the varying need and probability for membrane association. Palmitoylation in the cytosol is important when regulating the capability of proteins to diffuse freely or associate with the membrane. Intravesicular proteins cannot diffuse long distances and they are constantly relatively close to the membrane. Transmembrane proteins also undergo palmitoylation at their cytosolic side rather than at their vesicular side. The reason for this could be speculated to be associated with redundancy in the regulation of protein palmitoylation; the substrate specifities of the DHHC PAT proteins show some overlap at least in yeast (Roth et al. 2006). Further, the depalmitoylation of transmembrane proteins from the cytosolic side would not be dependent on the location of the palmitoylprotein.

2.1.4 Depalmitoylation

While several different palmitoyl transferases have been identified recently, current knowledge of depalmitoylating enzymes is somewhat limited (Huang and El-Husseini 2005). So far, only two enzymes possessing depalmitoylation activity on proteins, acyl protein thioesterase 1 (APT1) and palmitoyl protein thioesterase 1 (PPT1) (Lu and Hofmann 2006), the subject of this thesis, are known. PPT2, a homolog of PPT1, cleaves thioester bonds from linear molecules such as S-palmitoyl-CoA, but is unable to cleave palmitate from substrates with bulky head groups such as palmitoylated proteins (Calero et al. 2003). While PPT1 has been shown to function in lysosomes (Lu et al. 1996), APT1 operates in the cytosol (Duncan and Gilman 1998). It is worth mentioning that when APT1 was purified from rat liver, no palmitoyl thioesterase activity against palmitoyl-Gα was detected other than those derived from PPT1 and ATP1 (Duncan and Gilman 1998).

The crystal structure of APT1 has been determined and it belongs to a large family of α/β hydrolases, as does PPT1 (Devedjiev et al. 2000). APT1 is a dimer and it has to dissociate to reveal the substrate binding site. APT1 removes palmitate from several substrates *in vitro*, including H-Ras, Gsα, and endothelial nitric oxide synthase (eNOS) (Yeh et al. 1999). Depalmitoylation of these proteins by APT1 is more efficient when the substrates are activated (Smotrys and Linder 2004). APT1

deficiency in humans has not been reported, but yeast *apt1* null cells grow normally and do not show defects in lipid metabolism although they lack acyl-protein thioesterase activity (Duncan and Gilman 2002). In murine small intestine epithelial cells, overexpression of APT1 led to translocation of mutant Gαs (involved in a neonatal diarrhea phenotype) from the plasma membrane to the cytosol and reduced cAMP levels (Makita et al. 2007). Interestingly, based on the sequence in mass spectrometry, another acyl-protein thioesterase APT2 was reported to be associated with membranes and especially lipid rafts in HeLa cells, but its function *in vivo* has not yet been reported (Foster et al. 2003).

2.1.5 Palmitoylation and neuronal function

Protein palmitoylation is especially abundant in neurons. It is involved in a variety of neuronal functions, including neuronal development, synapse maturation, signal transduction, and intracellular trafficking. The dynamic nature of palmitoylation is particularly important in neurons, providing means to respond rapidly to external stimuli, and in controlling neuronal growth, differentiation, and plasticity. The first axonal palmitoylprotein described was the growth-associated protein 43 (GAP-43), a cytosolic phosphoprotein interacting with the actin cytoskeleton and regulating the outgrowth and pathfinding of axons (Skene and Virag 1989, Strittmatter et al. 1992). GAP-43 possesses two S-palmitoylations near the amino terminus, enabling the protein's attachment to the growth cone plasma membrane and regulating its function as a modulator of the G-protein signalling cascade (Sudo et al. 1992). Synapse maturation is induced by a specific decrease in the palmitoylation of GAP-43 and other growth cone proteins at the beginning of the critical period of synaptic rearrangement (Patterson and Skene 1999). On the postsynaptic side of excitatory synapses, a scaffolding molecule, postsynaptic density-95 (PSD-95) protein, is needed for the correct assembly of AMPA-type glutamate receptors and cell adhesion molecules (Kim and Sheng 2004). The dual palmitoylation of PSD-95 at its N-terminal end is required for its targeting and clustering at the postsynaptic density (Craven et al. 1999). Synaptic activity regulates the palmitate turnover of PSD-95; agonist-stimulated depalmitoylation of PSD-95 enhances AMPA receptor internalization (El-Husseini Ael et al. 2002) and disruption of PSD-95 palmitoylation blocks synaptic strengthening (Ehrlich and Malinow 2004).

Neuronal signal transduction is initiated by the release of neurotransmitters, which bind to their receptors and activate downstream effectors. Synaptic vesicles, containing neurotransmitters, release their cargo to the synaptic cleft upon fusion with the presynaptic plasma membrane in a process called exocytosis. Several presynaptic proteins controlling synaptic vesicle exocytosis are palmitoylated,

including synaptotagmin I, the calcium sensor for neurotransmission, and the SNARE proteins VAMP (vesicle associated membrane protein) and SNAP25 (synaptosome associated protein 25), which regulate membrane fusion. Palmitoylation controls the axonal targeting of synaptotagmin I, while palmitoylation of SNAP25 is needed for its function in synaptic vesicle dissociation but not for its targeting (Heindel et al. 2003, Washbourne et al. 2001). Neurotransmitters such as GABA (γ-aminobutyric acid), glutamate, dopamine, and serotonine all signal through G-protein coupled receptors. Similarly to the previously mentioned Gα proteins, many of the receptors coupled to them are also palmitoylated. For example, yeast-two-hybrid studies showed that a DHHC-protein GODZ palmitoylates the $GABA_A$ receptor and regulates its clustering to the postsynaptic membrane (Keller et al. 2004). Palmitoylation is important not only for targeting the receptor to specific membrane domains (Qanbar and Bouvier 2003), but depending on the receptor, it also up- or downregulates downstream G-protein signalling (el-Husseini Ael and Bredt 2002).

An example of severe neuronal dysfunction caused by the disruption of protein palmitoylation is the autosomal dominant Huntington's disease, characterized by involuntary body movements (chorea) and cognitive disability. It is caused by polyglutamine (CAG) repeat expansions in the huntingtin gene, making the encoded huntingtin protein prone to aggregation (Landles and Bates 2004). Huntingtin, among many other neuronal proteins, was found to be palmitoylated by the DHHC-protein HIP14 (huntingtin interacting protein 14) (Huang et al. 2004). Huntingtin functions in microtubule-mediated transport, specifically enhancing the trafficking of vesicles containing brain-derived neurotrophic factor (BDNF) (Gauthier et al. 2004). Palmitoylation regulates the intracellular trafficking and function of huntingtin, and mutations expanding the polyglutamine tract disturbed the interaction between huntingtin and HIP14, and resulted in decreased palmitoylation and increased the aggregation of huntingtin (Yanai et al. 2006). Palmitoylation and its regulation have been proven to be vital for the function of many neuronal proteins, and understanding the involved mechanisms will help to elucidate neuronal events in health and disease.

2.2 LYSOSOMES

2.2.1 Intracellular membrane transport

Eukaryotic cells are compartmentalized into several membrane-bound organelles with distinct functions and unique protein and lipid compositions. These organelles are linked to each other by constant trafficking, where vesicles bud from one

compartment and fuse with another. In order to achieve and maintain these highly specified compartments, a strictly controlled transport system has developed to select the soluble protein cargo, the bilayer of lipids, and the membrane proteins of the transport vesicle. Similarly, the uptake of the cargo at its destination is tightly regulated. Independent of the cellular location, vesicular trafficking consists of five elementary steps: 1) sorting of proteins and lipids to be transported, 2) vesicle formation, 3) vesicle transport along cytoskeletal filaments, 4) target organelle recognition, and 5) vesicle fusion with the target organelle. Proteins often require "address labels" to be correctly targeted, while lipids seem to hold the information about their target in their structure (Bonifacino and Glick 2004, Mellman and Warren 2000, Olkkonen and Ikonen 2000, Roux et al. 2005). Disturbances in the transport machinery underlie at least 30 human diseases. Most of them exhibit a neurological phenotype and specifically concern the movement of vesicles along the cytoskeletal tracts. This emphasises the importance of intracellular membrane trafficking for the correct function of neurons, and suggests membrane trafficking to have redundancy in most cell types (Olkkonen and Ikonen 2006).

Intracellular membrane trafficking consists of two main pathways; the secretory or the biosynthetic pathway, and the endocytic pathway (**Figure 3**). The constitutive secretory pathway is used for the secretion of produced compounds (proteins, lipids, and carbohydrates) from the endoplasmic reticulum (ER) and Golgi complex to the cell surface and extracellular space. The secretory pathway is also used for targeting the products to endosomes and lysosomes via the Golgi complex. The endocytic pathway covers internalization of macromolecules, for example proteins, ligands, and receptors, as well as solutes and pathogens. After internalization, the cargo is transported to early endosomes, from where it can be recycled back to the cell surface via recycling endosomes. Cargo can also enter the degradative pathway and be transported to multivesicular bodies, late endosomes, or lysosomes. The secretory and endocytic pathways are connected via the Golgi complex and the endocytic compartments (Bonifacino and Rojas 2006, Nickel and Wieland 1998, van der Goot and Gruenberg 2006).

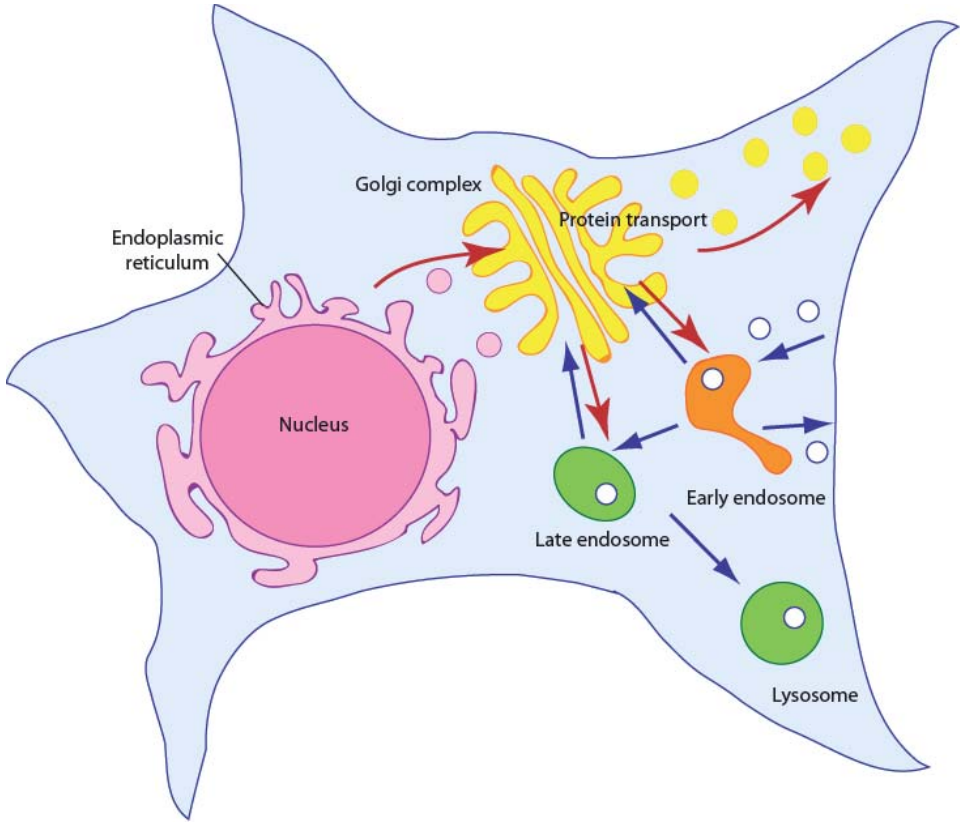

Figure 3. ***Pathways of intracellular membrane transport. The secretory pathway (red arrows) transports cargo from the endoplasmic reticulum (ER) through the Golgi complex to the plasma membrane or to the endocytic organelles. Cargo internalized from the extracellular space (blue arrows) enters the early endosomes and may be recycled or transported to late endosomes and lysosomes. The two pathways are connected at the level of the Golgi complex and the endosomal organelles. Figure modified from*** (Olkkonen and Ikonen 2000)***.***

~~2.2.2 Structure and function of lysosome~~

Upon their discovery, lysosomes (Greek for digestive body) were classified as acid hydrolase-rich membrane-limited granules (De Duve et al. 1955). Indeed, the terminal degradation of proteins and lipids occurs in lysosomes, but over 50 years of research has proved that lysosomes are also physiologically important in other functions than merely catabolism. These include, for example, surface receptor

downregulation, release of endocytosed nutritients, and inactivation of pathogens (Eskelinen et al. 2003).

The limiting membrane of the lysosome contains special features important for its function. Low lysosomal pH (<5) is necessary for the activation of most lysosomal degradative enzymes, and it is gained and maintained through an active pumping of protons into the organelle by a multimeric transmembrane protein, the vesicular (v-) H^+-ATPase. The v-H^+-ATPase also mediates the information about intravesicular pH to the cytosolic surface of the vesicle, which in turn is important for the maturation and trafficking of endosomes (Marshansky 2007). The lysosome-associated membrane proteins (LAMPs) 1 and 2 make up about 50% of all proteins ín the lysosomal membrane. They are highly glycosylated and, together with LIMPs (lysosomal integral membrane proteins) and other glycosylated membrane proteins, they form a protective glycocalyx intralumenally on the limiting membrane. Emerging data suggest that they have other functions as well; for example LAMPs are required for the fusion of lysosomes and phagosomes (Huynh et al. 2007) and LIMP-2 has recently been shown to function as a receptor for lysosomal targeting (Reczek et al. 2007). Other, minor lysosomal membrane proteins are responsible for the transport of metabolites such as sugars, amino acids, cholesterol, and metals across the lysosomal membrane to cytosol.

The lysosomal degradation of macromolecules occurs in a sequential manner and involves the action of over 50 hydrolytic enzymes and associated proteins (Sleat et al. 2005). For example, the degradation of glycans linked to glycoproteins takes place in several sequential steps, all catalyzed by specific enzymes (Winchester 2005). The degradation of membrane lipids occurs on the surface of intra-endosomal and intra-lysosomal vesicles which are formed after inward budding of the limiting membrane (**Figure 4**) (Hopkins et al. 1990). Membranes to be digested are cholesterol-poor compared to the limiting membrane, and rich in negatively charged bis(monoacylglycero)phosphate (BMP), also known as lysobisphosphatic acid (LBPA) (Mobius et al. 2003). While other lipids (e.g. phospholipids) do not need cofactors for their degradation, glycosphingolipids require sphingolipid activator proteins (SAPs), namely saposins and the GM2-activator protein, in order to be degraded. Glycosphingolipids, composed of ceramide and a hydrophilic oligosaccharide chain, are an essential part of the outer leaflet of the plasma membrane. In neuronal cells, glycosphingolipids containing sialic acid (ganglio-series) are abundant, while oligodendrocytes are rich in galactosylceramide (Kolter and Sandhoff 2005). Saposins A, B, C, and D are small ~80-aa lysosomal glycoproteins derived from a common precursor protein called prosaposin. They stimulate specific lysosomal sphingolipid hydrolases including ceramidases, arylsulfatase A, sphingomyelinase, and galactosidase (Kishimoto et al. 1992). They

also have lipid transfer activity *in vitro* and they participate in immunological events by loading antigenic lipids to CD1 proteins (Kang and Cresswell 2004).

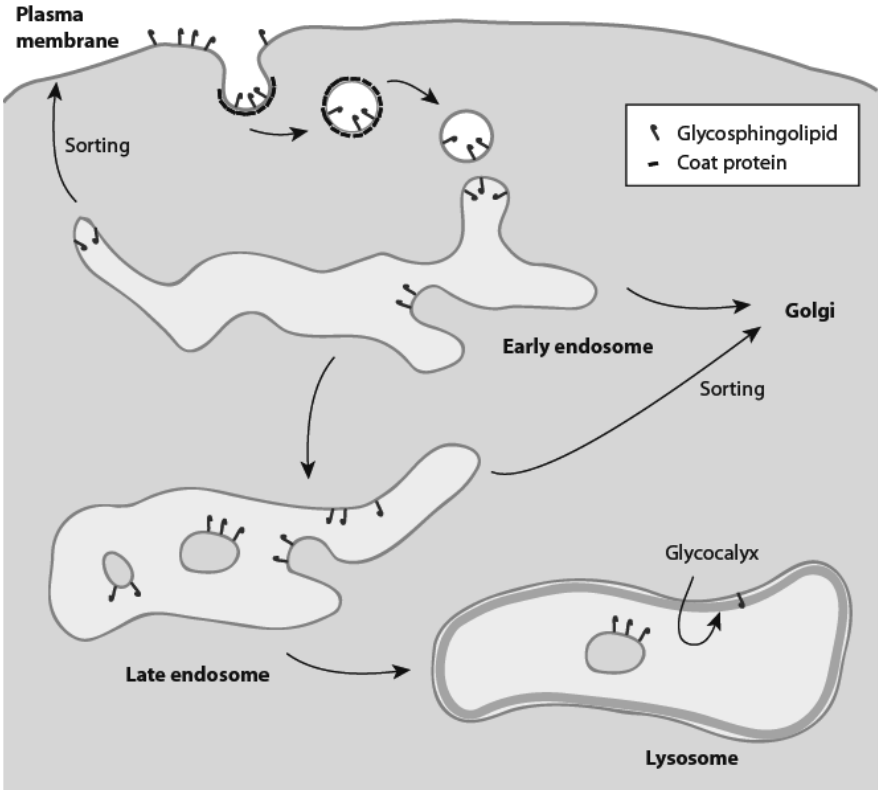

Figure 4. ***Schematic representation of membrane digestion in lysosomes. Glycosphingolipids are shown on the plasma membrane and on internal membranes. The intra-endosomal membranes act as platforms for the degradation of glycosphingolipids. The inner face of the lysosomal limiting membrane is protected from degradation by a glycocalyx. Figure modified from*** (Kolter and Sandhoff 2005)**.**

2.2.3 Lysosomal targeting of soluble proteins

Lysosomes receive cargo from the secretory, endocytic, autophagic, and phagocytic membrane transport pathways (Luzio et al. 2007). Intracellular transport of newly synthesized proteins from the secretory pathway to the endocytic pathway, via the

endosomes to the lysosomes, is probably the best characterized (Kornfeld and Mellman 1989). Synchronously with their synthesis, soluble lysosomal proteins are translocated to the ER, where their signal sequence is cleaved and they are folded and glycosylated at the asparagine residues. From the ER, the proteins are targeted to the Golgi complex, where the oligosaccharides are trimmed and they receive the specific carbohydrate modification for lysosomal targeting, the mannose 6-phosphate (Man-6-P). Man-6-phosphorylation occurs in two steps: First, N-acetylglucosamine-1-phosphotransferase adds a N-acetylglucosamine-phosphate group to the mannose residues, followed by cleavage of the N-acetylglucosamine from the phosphate by N-acetylglucosamine-1-phosphodiester α-N-acetylglucos-aminidase (Hasilik et al. 1981, Waheed et al. 1981).

In the *trans*-Golgi network (TGN), mannose 6-phosphorylated proteins are recognized by one of the two Man-6-P receptors (MPRs), the cation-dependent 46 kDa MPR (CD-MPR) or the cation-independent 300 kDa MPR (CI-MPR). MPRs, together with their ligands, are packed into clathrin-coated vesicles. Clathrin adaptor protein 1 (AP-1) and GGAs (Golgi-localized, γ-ear-containing, ADP ribosylation factor-binding proteins) are needed to sort the MPR to the clathrin vesicles in the TGN (Doray et al. 2002). After transport and uncoating, the intermediate vesicles fuse with the target organelle, an early or late endosome. A subsequent decrease in pH facilitates MPR ligands to dissociate from their receptors, and the MPRs are transported back to the TGN while the protein cargo reaches the lysosomes by the fusion of late endosomes and lysosomes (Luzio et al. 2007). Lysosomes lack both MPRs, differing thus from endosomes. Around one third of the MPRs in the cell are present at the plasma membrane, and are capable of binding to mannose 6-phophorylated proteins secreted either from the same or adjacent cells. MPRs and their ligands are endocytosed with the help of clathrin and AP-2 adaptor protein from the plasma membrane (Le Borgne and Hoflack 1998).

A second route to lysosomes for newly synthesized soluble hydrolases was detected in I-cell disease (mucolipidosis II) patient cells, where the N-acetylglucosaminidase-1-phosphotransferase enzyme is defective and lysosomal hydrolases do not receive the Man-6-P label needed for MPR recognition (Reitman et al. 1981). While most lysosomal proteases became secreted, nearly half of the normal amount of cathepsin D was still detected in the lysosomes of the patient cells (Glickman and Kornfeld 1993). The protein responsible for the MPR-independent lysosomal sorting was later found to be sortilin, a multi-ligand transmembrane Golgi protein similar to MPR. Sortilin also sorts other lysosomal proteins such as sphingolipid activator proteins (SAPs) to lysosomes (Lefrancois et al. 2003). It is involved in other cellular functions as well, as it mediates lipoprotein lipase degradation and translocates to the plasma membrane from GLUT4 glucose transporter-containing vesicles after

insulin-stimuli (Morris et al. 1998, Nielsen et al. 1999). A third sorting pathway to lysosomes was recently found for β-glucocerebrosidase, for which the lysosomal targeting signal was long unknown (Reczek et al. 2007). Lysosomal membrane protein LIMP-2 was found to bind with β-glucocerebrosidase already in the ER and target the proteins to lysosomes. The ER-retention mutant of β-glucocerebrosidase was also able to traffic to lysosomes when LIMP-2 was overexpressed.

2.2.4 Lysosomal storage disorders affecting the central nervous system

Lysosomal storage disorders (LSDs) are characterized by the intra-lysosomal accumulation of undegraded metabolites, caused by the disrupted function of lysosomal proteins. Over 40 LSDs are known, and most of them are caused by the deficiencies of soluble lysosomal hydrolases. Lysosomal transporters and integral membrane proteins are also involved, as are proteins that reside earlier in the secretory pathway and which affect the transport and maturation of lysosomal proteins (Futerman and van Meer 2004, Tardy et al. 2004). LSDs are often classified according to the main storage compound (e.g. sphingolipidoses and mucopolysaccharidoses). However, in some cases this has been misleading – for example, I-cell disease was first classified as mucolipidosis, although the defect is in the transport of lysosomal enzymes rather than in lipases themselves. The affected cellular pathways that underlie the cellular damage in LSDs are mostly unknown, as is the role of storage itself in the disease course.

LSDs present variable disease phenotypes, ranging from infantile to adult forms. In the more severe infantile forms neurological symptoms are often prominent, whereas peripheral dysfunction dominates in adult forms (Futerman and van Meer 2004). Neuronal ceroid lipofuscinoses, discussed later in this thesis, are LSDs characterized by severe neurodegeneration. An example of another LSD showing neurological manifestations is mucolipidosis type IV (MLIV), which is enriched in the Ashkenazi Jewish population. The symptoms include psychomotor retardation and ophthalmologic abnormalities. Mutations in the mucolipin-1-encoding gene *MCOLN1* result in the lysosomal accumulation of lipids together with water-soluble substances (Bargal et al. 2000). Mucolipin-1 is a cation channel and its dysfunction is suggested to lead to abnormal endocytosis and lysosomal maturation (Bach 2005, Zeevi et al. 2007). The lysosomal accumulation of free sialic acid results in two phenotypically different diseases with neurological manifestations, a severe infantile sialic acid storade disease (ISSD) and a slowly progessing adult form, Salla disease, which is enriched in Finland. Both diseases are caused by mutations in the *SLC17A5* gene, disrupting the function of sialin, an anion transporter residing in the lysosomal membrane (Verheijen et al. 1999).

LSDs are monogenic disorders, but the type of mutation and its location in the affected gene may vary. When the mutated protein is an enzyme, there can be some correlation between the residual enzyme activity and the disease phenotype (Gieselmann 2005). However, the phenotype can rarely be predicted based on the genotype. Sometimes the same mutation can result in exact opposite phenotypes, as is the case in Gaucher disease, a sphingolipodosis caused by mutations in β-glucocerebrosidase. An individual diagnosed with Gaucher disease can suffer from severe disease, while another individual with the same genotype may be asymptomatic (Beutler 2007). The reason for the phenotypic variance is not known, but environmental factors as well as modifying genes have been suggested to influence the clinical course.

2.2.5 Lysosomal proteins functioning outside lysosomes

Some lysosomal proteins have been shown to also operate extralysosomally. These include cathepsin D, the lysosomal protease defective in congenital NCL (Siintola et al. 2006), and β-hexosaminidase A, the enzyme disrupted in Tay-Sachs disease (Beutler et al. 1975). Cathepsin D is synthesized as a prepropeptide and is proteolytically modified into its active form. The pre-peptide acts as a signal sequence and is removed after translocation to the ER. Procathepsin D is inactive, until the pro-peptide is cleaved in the endosomal or lysosomal compartments (Erickson et al. 1981). The increased secretion of the inactive pro-enzyme has been implicated in many types of cancer, presumably enhancing the proliferation, invasion, and metastasis of cancer cells (Fusek and Vetvicka 1994). The pro-peptide has been shown to possess mitogenic activity, being independent of MPR but functioning through some yet undefined cell surface receptor (Ohri et al. 2008). An additional role for a lysosomal enzyme, independent of its activity or correct lysosomal trafficking, is thus present in paracrine signalling.

β-hexosaminidase A and B are isoenzymes cleaving glucosamine- and galactosamine-N-acetyl residues from oligosaccharides, glycoproteins, and glycolipids. β-hexosaminidase A (Hex A) can specifically cleave GM2 ganglioside, concentrated in lipid rafts and on the neuronal cell surface, in the presence of sphingolipid activator protein GM2-activator (Wendeler et al. 2004). Hex A is mainly found as a soluble enzyme inside the lysosomes where it is targeted via the MPR pathway and processed into its mature form (Mahuran 1999). A recent study reports 3-5% of mature Hex A to be present on the cell surface, as shown by cell surface biotinylation and activity assays (Mencarelli et al. 2005). This finding suggests an exchange of Hex A via a direct fusion between lysosomes and plasma membrane, a phenomenon described for example in plasma membrane repair

(Reddy et al. 2001). In addition, the Hex A enzyme was found to be membrane-associated, although no structural evidence for this is found in the enzyme itself. Hex A may thus have a role outside lysosomes, for example possibly being capable of modifying the lipid composition of the outer leaflet of the neuronal plasma membrane and thus further influencing the signalling processes of the cell. These findings highlight the importance of the possible additional functions of lysosomal enzymes in physiological and pathological processes and may in part explain the phenotypic variance seen in many LSDs.

2.3 CENTRAL NERVOUS SYSTEM AND CHOLESTEROL METABOLISM

2.3.1 Brain and cholesterol homeostasis

Brain tissue is composed of signalling nerve cells, or neurons, and glial cells, namely astrocytes, oligodendrocytes, and microglia. Glial (Greek for glue) cells, comprising ~90% of the total cell count in the central nervous system (CNS), were initially thought to be the "connective tissue" of the brain (Gray 1918). Today, glial cells are known to be more versatile and they have proven to be an essential part of the correct functioning of the nervous system. Oligodendrocytes provide insulation for the electricity-conducting axons of neurons by forming a myelin sheath around them (Simons and Trotter 2007). Microglia are the "immune system of the brain" and respond to brain injury and immunological stimuli (Hanisch and Kettenmann 2007). Astrocytes regulate blood flow in the brain, modulate synaptic function, and transport nutritients to neurons (Maragakis and Rothstein 2006, Voutsinos-Porche et al. 2003). They are also central players in the cholesterol metabolism of the CNS (Mauch et al. 2001). Systemic cholesterol metabolism has been widely studied because of its well-established role in atherosclerosis. Cholesterol metabolism in the CNS, however, has only recently been elucidated. Cholesterol, together with phospholipids, is an essential lipidous part of the plasma membrane in human cells. The CNS is particularly rich in cholesterol, and the cholesterol concentration in the brain is around ten times higher than in other organs. Most of the cholesterol in the CNS is stored in myelin, as myelin is composed 70% of lipids enriched with cholesterol and galactosylceramide (cerebroside) (Simons and Trotter 2007). While cholesterol in other parts of the body can either be synthesized *de novo* or taken up from plasma, the CNS is isolated from plasma cholesterol supplies by the blood-brain barrier (BBB) and is self-sustaining in cholesterol (Dietschy and Turley 2001). The rate of cholesterol synthesis in the brain during development follows the production of myelin by oligodendrocytes, which is highest during the first 4 weeks

of life in mice (the first and early second years of life in the human) (Brody et al. 1987). The rate of cholesterol synthesis declines after myelination, but remains at a steady level, reflecting synthesis in other parts of the CNS (Dietschy and Turley 2004). Excess cholesterol in the brain is removed by converting it to 24(*S*)-hydroxycholesterol, which is able to cross the BBB (Lutjohann et al. 1996). Cholesterol 24-hydroxylation is performed by the enzyme CYP46A1, which resides exclusively in neurons (Lund et al. 1999).

2.3.2 Lipoproteins in plasma and cerebrospinal fluid

Lipoproteins are macromolecular complexes composed of lipids and proteins called apolipoproteins. In plasma, they transport lipids from one part of the body to another, providing peripheral cells with energy, precursors to steroids, and material for membrane synthesis. They also transport lipids from peripheral cells especially via the HDL-facilitated pathway called reverse cholesterol transport, whereby cholesterol enters the liver for excretion or for use in bile acid synthesis. Lipoprotein particles are composed of a neutral lipid core containing triacylglycerols and esterified cholesterol, and a polar surface composed of phospholipids and apolipoproteins. Lipoprotein particles are traditionally classified according to their densities, and named consequentially: HDL, or high-; LDL, or low-; and VLDL, or very low-density lipoprotein. These three classes are the major ones found in the circulation. Basically, the more neutral lipids the particle carries, the lower the density. Apolipoproteins play a central role in lipoprotein metabolism. They are located on the surface of the particle and are capable of binding to receptors on the cell surface, directing lipoprotein metabolism. They are also important cofactors for some key enzymes in lipid metabolism (Danik et al. 1999, Gotto et al. 1986). In addition to plasma, lipoproteins are found also in cerebrospinal fluid (CSF), where they transport lipids between different cell types of the nervous system. The lipoproteins in CSF differ from those in plasma in size and apolipoprotein composition. In plasma, the main lipoprotein particles are spherical and the major apolipoproteins linked to VLDL are apoB-100 and apoE, whereas LDL is mostly linked to apoB-100 and HDL to apoA-I. The main apolipoproteins in CSF are apoE, apoJ and apoA-I, and the HDL-like lipoprotein particles are discoidal and smaller than those in plasma (Koch et al. 2001, Legleiter et al. 2004). ApoE is synthesized mainly by astrocytes (Pitas et al. 1987) and microglia (Nakai et al. 1996), whereas apoA-I is not synthesized in the CNS. It is thought to either cross the BBB via transcytosis through capillary endothelial cells or to be synthesized by these cells (Kratzer et al. 2007, Panzenboeck et al. 2002).

2.3.3 Cholesterol transport in the CNS

Although current knowledge about cholesterol trafficking inside the CNS is limited, many proteins involved in plasma cholesterol metabolism are also expressed in the CNS, suggesting the existence of similar pathways. In addition to apolipoproteins (mainly apoE) expressed by astrocytes, the CNS cells also express lipoprotein receptors belonging to the LDL receptor (LDLR) family. These structurally related receptors have diverse functions and ligands, being involved in Wnt and Reelin signalling and intracellular transport in addition to their function in cholesterol transport (Cam and Bu 2006). The LDLR family consists of over 10 members, including LDLR, VLDLR, LRPs (LDL receptor –related proteins), apoER2, sorLA (sortilin–related receptor containing LDLR class A repeats, also called LR11), and they all bind apoE. Neurons express many of these receptors, especially LRP1, VLDLR, apoER2, and sorLA (Andersen and Willnow 2006), whereas astrocytes express mostly LDLR (Rapp et al. 2006). ATP-binding-cassette transporters (ABC transporters), involved in the transport of lipids and other substances across the membranes, are also expressed in different cell types of the CNS. Especially ABCA1, ABCA2, ABCG1, and ABCG4 are abundant in the brain (Kim et al. 2008). Transmembrane ABC transporters differ in their intracellular localization and the type of transported lipids, as well as in the type of the lipid acceptor protein. ABCA1, residing mostly in the plasma membrane, mediates cholesterol efflux from neurons to the lipid-poor apoE and apoE-phospholipid discs (Kim et al. 2007). Endo/lysosomal ABCA2 is mostly expressed by oligodendrocytes and is involved in intracellular sphingolipid transport and myelin maturation (Sakai et al. 2007). Vesicular ABCG1 and ABCG4 stimulate cholesterol efflux and mediate intracellular cholesterol transport in neurons and astrocytes (Tarr and Edwards 2008, Wang et al. 2004).

Like other cells, neurons also are able to synthesize cholesterol. During synaptogenesis, however, they are dependent on external cholesterol from glia-derived apoE-containing lipoproteins. Cholesterol increases the number of formed synapses and its function is inhibited by blocking the members of the LDLR family (Mauch et al. 2001). More specifically, cholesterol has been found to directly promote maturation of the presynaptic terminals and to be important in dendrite differentiation (Goritz et al. 2005). It was later also found that not all neurons are alike; while retinal ganglion cells need glia for synapse formation, hippocampal and cerebellar neurons efficiently form synapses without astrocytes (Steinmetz et al. 2006). Also, excitatory synapses are enhanced by glial cholesterol in all neuron types, while it has no effect on inhibitory synapses. Studies on hippocampal astrocytes and neurons showed that neurons are more dependent on the apoE-bound cholesterol than astrocytes, and apoE-derived cholesterol was associated with membranes significantly more in neurons than in astrocytes (Rapp et al. 2006).

ApoE expression is also strongly increased during regeneration followed nerve injury, further indicating its essential role in the membrane repair of neurons (Poirier et al. 1993).

2.3.4 Cholesterol and neurodegenerative diseases

The importance of cholesterol metabolism for CNS function is underlined by its connection with severe neurological diseases such as Alzheimer's disease (AD) and Niemann-Pick type C (NPC). AD is characterized histopathologically by the formation of extracellular amyloid plaques composed of β-amyloid peptide (Aβ) and intracellular neurofibrillary tangles consisting of abnormally phosphorylated tau protein. Aβ is formed by the sequential proteolytic cleavage of the amyloid precursor protein (APP) by β- and γ-secretases (Kang et al. 1987, Zhang and Xu 2007). The link between AD and cholesterol was first noted in population-based studies when the *APOE4* isoform was found to be associated with increased risk of late-onset AD (Saunders et al. 1993, Schmechel et al. 1993). Hypercholesterolemia itself was found to be an independent risk factor for AD (Kivipelto et al. 2001), and cholesterol is needed for Aβ formation in hippocampal neurons (Simons et al. 1998). Therefore statins, drugs that inhibit HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase activity and reduce cholesterol synthesis, have been studied in the prevention of AD; however, the results have been controversial (Canevari and Clark 2007). At the moment it is not evident how apoE4, cholesterol, and the development of AD are interconnected, but intensive research has revealed many contributing cellular mechanisms. Cholesterol bound to the apoE4-phospholipid complexes could not be taken up by neurons as efficiently as cholesterol bound to other apoE isoforms (Rapp et al. 2006). When apoE-containing lipoproteins were shown to protect CNS neurons from apoptosis by the LRP-receptor-mediated pathway, apoE4 was less protective than apoE3 (Hayashi et al. 2007). ApoE is also important for the Aβ clearance (Ladu et al. 2000). Many LDLR family members have been shown to interact with APP and regulate its endocytic processing, thus influencing the formation of Aβ (Cam and Bu 2006). The role of lipids other than cholesterol in AD has also been implicated. Sphingomyelin prevented Aβ formation and sphingomyelinase activity is increased in some familial forms of AD (Grimm et al. 2005). A diet rich in polyunsaturated fatty acids, for example regular fish consumption, decreases the risk of AD (Morris et al. 2003). At least one reason for this has been reported: omega-3 fatty acid docosahexaenoic acid (DHA), present in fish oil, significantly increased the expression of SorLA, a LDLR protein involved in Aβ reduction (Ma et al. 2007).

NPC is an autosomal recessive lysosomal storage disorder caused by mutations in two genes, NPC1 and NPC2 (Carstea et al. 1997, Naureckiene et al. 2000). The precise functions of the NPC proteins are not known, but they are thought to have a role in intracellular cholesterol transport as the disease is characterized by the accumulation of free cholesterol, bis(monoacylglycero)phosphate (BMP), and sphingolipids in the endo/lysosomal compartments of the cell (Ikonen and Holtta-Vuori 2004). Clinical symptoms are diverse, but neurological impairment is one of the disease hallmarks. Npc1-deficient glial cells accumulate cholesterol, but their function appears normal and they generate normal apoE-containing lipoproteins. In Npc1-deficient neurons, however, the distribution of intracellular cholesterol is altered, and cholesterol transport from the cell soma to axons is inhibited (Vance et al. 2006). Cholesterol and sphingolipids are both typical components of lipid rafts and NPC1 has been shown to participate in the depletion of lipid rafts from late endocytic organelles (Lusa et al. 2001). It has been suggested that cholesterol accumulation could actually be secondary to sphingolipidosis, as sphingolipids are the primary storage compound in neurons and are needed for cholesterol accumulation (Gondre-Lewis et al. 2003).

2.4 NEURONAL CEROID LIPOFUSCINOSES

2.4.1 Common features in NCLs

Neuronal ceroid lipofuscinoses (NCLs) are a group of autosomal, recessively inherited progressive encephalopathies with an estimated incidence of 1:12 500 in the United States (Rider and Rider 1988). The diseases can have congenital, infantile, late infantile, juvenile, or adult onset. The childhood forms present the majority of NCLs, and NCLs are considered to constitute the most common group of neurodegenerative diseases in children worldwide (Haltia 2003). The early-onset diseases share similar clinical symptoms such as loss of vision, mental and motor deterioration, epileptic seizures, and premature death (Santavuori et al. 2000). The first symptoms in adult NCLs are usually cognitive impairment and early dementia (Hinkebein and Callahan 1997, van Diggelen et al. 2001). Symptoms result from severe neuronal death occurring mainly in the cerebral and cerebellar cortices.

In addition to neurodegeneration, histopathological findings are also common to all NCL subtypes. Neurons, and many other cell types, accumulate autofluorescent storage material called ceroid-lipofuscin (Zeman and Dyken 1969). Storage deposits are resistant to lipid solvents and their ultrastructural phenotype is an important classification method before possible genetic tests (Goebel 1997). In most NCLs, over 50% of the storage material consists of subunit c of the mitochondrial ATP

synthase (Palmer et al. 1992). In infantile and congenital NCLs the main storage compounds are sphingolipid activator proteins (SAPs) (Tyynela et al. 1993). The main stored compound seems to determine the storage ultrastructure – SAPs are associated with GROD-like structures (granular osmiophilic deposits), while the storage of subunit c of ATP synthase results in more variable forms of curvilinear, rectilinear, or fingerprint patterns (Haltia 2006).

To date, over 200 mutations in eight different genes have been identified behind the NCL diseases (*CLN1*, *CLN2*, *CLN3*, *CLN5*, *CLN6*, *CLN7, CLN8* and *CLN10/CTSD*) (NCL Resource database, http://www.ucl.ac.uk/ncl/mutation.shtml, S. Mole) (Siintola et al. 2007, Siintola et al. 2006) and they serve as the basis for the current NCL classification. A summary of the genes and the disease phenotypes, affected proteins, storage material, and ultrastructure related to them are shown in **Table 1**. INCL and vLINCL$_{Fin}$, caused by mutations in the *CLN1* and *CLN5* genes, respectively, are discussed later in more detail.

2.4.2 NCLs in animals

NCLs are not merely a human disease, as different NCLs also affect many animals. These include sheep, with mutations in the *CTSD*, *CLN5,* and *CLN6* genes (Broom et al. 1998, Frugier et al. 2008, Houweling et al. 2006, Tyynela et al. 2000). Dogs are affected with mutations in *CLN2*, *CLN8*, *CLN5,* and *CTSD* (Awano et al. 2006a, Awano et al. 2006b, Katz et al. 2005, Melville et al. 2005b). Cattle with the *CLN5* mutation have also been reported (Houweling et al. 2006). Cats, pigs, birds, and horses have been reported to suffer from NCL as well (Goebel et al. 1999). A naturally occurring large animal model with PPT1 deficiency remains to be characterized.

Table 1. ***Classification of the NCLs. Disease phenotypes, affected proteins, storage ultrastructure, and the main storage material are presented. GROD, granular osmiophilic deposits; CL, curvilinear profiles; FP, fingerprint bodies; RL, rectilinear complexes.***

Gene	Disease	Protein	Storage ultra-structure	Main storage material
CLN1	Infantile, also late infantile, juvenile and adult	PPT1, palmitoyl protein thioesterase 1, soluble	GROD	Saposins A and D
CLN2	Late infantile, juvenile	TPP1, tripeptidyl peptidase 1, soluble	CL	Subunit c of ATP synthase
CLN3	Juvenile	CLN3, transmembrane	FP (CL, RL)	Subunit c of ATP synthase
CLN4 (not identified)	Adult	Not known	FP, granular	Subunit c of ATP synthase
CLN5	Late infantile, Finnish variant	CLN5, soluble	RL, CL, FP	Subunit c of ATP synthase
CLN6	Late infantile	CLN6, transmembrane	RL, CL, FP	Subunit c of ATP synthase
CLN7 (MFSD8)	Late infantile, Turkish variant	MFSD8, transmembrane	RL, CL, FP	Subunit c of ATP synthase
CLN8	Late infantile, Northern epilepsy	CLN8, transmembrane	CL	Subunit c of ATP synthase
CLN9 (not identified)	Juvenile	Not known	CL (FP, GROD)	Subunit c of ATP synthase
CTSD (CLN10)	Congenital, late infantile	CTSD, Cathepsin D, soluble	GROD	Saposins A and D

2.4.3 Mitochondria and NCLs

The role of mitochondria in the pathogenesis of NCLs has been under investigation for over a decade. The reason for this is the accumulation of subunit c of the mitochondrial ATP synthase in most NCLs. Neurons are extremely vulnerable to energy depletion resulting from mitochondrial dysfunction. Increasing evidence also

suggests that mitochondria, the important regulators of cell death, have a key role in the neurodegeneration of aging-related diseases via apoptosis and oxidative stress (Lin and Beal 2006). Additionally, mutations in mtDNA or nuclear genes encoding mitochondrial proteins lead to mitochondrial diseases of which neurological impairment is a hallmark (Zeviani and Carelli 2007).

Subunit c of the ATP synthase accumulates in the storage deposits in 8 out of 10 NCLs (**Table 1**). It is a highly hydrophobic protein locating in the inner membrane of mitochondria and belonging to the F_o-complex of the ATP synthase (complex V). Subunit c oligomerizes to form a proton channel, allowing protons to flow from the intermembrane space to the matrix and harnessing the energy for ATP synthesis produced by the F_1-complex. When released, subunit c has a tendency to self-aggregate with lipids, and once formed, these aggregates are relatively insoluble and protease-resistant (Elleder et al. 1995). It has been postulated that there is a specific problem in the catabolism of subunit c, since studies with ovine NCL showed neither defects in the amount or structure of subunit c in mitochondria nor defects in oxidative phosphorylation (Palmer et al. 1992). TPP1, the enzyme defective in LINCL (CLN2), has in fact been demonstrated to be associated with the degradation of subunit c (Ezaki et al. 1999). The activity of TPP1 is elevated in other NCLs accumulating subunit c, but also in INCL lacking subunit c storage (Junaid and Pullarkat 1999).

Autophagy, or cellular self-eating, is a lysosomal degradation pathway for the cytoplasm, i.e. cytosolic proteins and organelles (Eskelinen et al. 2005). Autophagy has proven to be an important pathway for cell survival – not only as a way for the cell to adapt to starvation, but also as a part of its normal homeostasis. Disturbances in the autophagic processes have been postulated to be involved in the pathogenesis of many diseases such as cancer, infectious diseases, and neurodegenerative diseases (Mizushima et al. 2008). Mouse models for JNCL and congenital NCL have been shown to possess induced autophagy, determined by the increased amounts of LC3-II (microtubule associated protein 1 light chain 3), an autophagosome-specific membrane-bound protein, in the nervous tissues of the mouse models. The predominance of immature autophagic structures in brain tissue was verified by cell fractionation and electron microscopy (Cao et al. 2006, Koike et al. 2005). Autophagosomes are double-membrane-bound vacuoles wrapped around cytosolic organelles, including mitochondria. Abnormal degradation of the autophagocytosed material could underlie the accumulation of subunit c with a typical storage ultrastructure, although the exact role of CLN3 or CTSD in autophagy is yet unknown.

Morphological changes in the mitochondria have been detected in some NCL subtypes. Abnormal mitochondria have been reported in JNCL patients (Zeman and

Donahue 1963). Cultured primary neurons derived from two different Cln3-deficient mouse models also show enlarged mitochondria (Fossale et al. 2004, Luiro et al. 2006). Enlarged mitochondria with abnormal cristae were also found in the GABAergic neurons of the English setter model for CLN8 (March et al. 1995). CLN6-affected sheep and Ppt1-deficient *C. elegans* (INCL model) show mitochondrial abnormalities in neurons (Porter et al. 2005, Walkley et al. 1995).

Mitochondrial dysfunction is also reported in several NCLs. Studies have been conducted with CLN1, CLN2, and CLN3 human patient and CLN6 sheep fibroblasts (Das et al. 1999, Das et al. 1996). Regulation of the mitochondrial ATP synthase was measured after exposing the cells to different stimuli including anoxia and calcium. The anomalies in ATP synthesis varied between diseases, but they were present in each disease type studied. The general conclusion was that disturbances in ATP production could be linked to energy-linked excitotoxicity, especially in metabolically active neurons (Jolly et al. 2002b). In this scenario, ATP deficiency would first lead to insufficient plasma membrane repolarization driven by the Na^+-K^+-ATPase pumps. This would further lead to the increased flow of Ca^{2+} in the cytoplasm followed by calcium-mediated neuronal death. In fact, Cln3-deficient mouse neurons showed slower recovery from depolarization when N-type calcium channels were blocked, potentially leading to Ca^{2+}-induced excitotoxicity (Luiro et al. 2006). Results from studies on Ppt1-deficient mouse neurons were quite opposite; the knock-out neurons recovered better from the glutamate-induced calcium peak than the control neurons. However, many genes involved in calcium buffering were also upregulated in Ppt1-deficient neurons, suggesting a possible attempt to correct the situation (Ahtiainen et al. 2007).

2.4.4 NCL proteins connected to lipids

CLN3, the protein defective in JNCL, is an endo-lysosomal transmembrane protein with a yet undefined function (Kyttala et al. 2004). However, two recent studies have implicated the involvement of CLN3 in lipid metabolism. First, Narayan and co-workers identified a low-stringency match in the N-terminal CLN3 sequence to a fatty acid desaturase domain (Narayan et al. 2006). They found that the CLN3 desaturase activity was the strongest towards palmitoylated proteins, turning palmitate into palmitoleic acid. This modification would affect the substrate's ability to locate, for example, in highly ordered lipid rafts. A second study reported an association of CLN3 with BMP synthesis (Hobert and Dawson 2007). Hobert and co-workers noted that detergent-resistant microdomains (DRMs or lipid rafts) extracted from JNCL patients' brains (autopsy samples) were less buoyant than the control samples. To explain this, they examined the phospholipid composition of

DRMs and found that BMP levels in CLN3-deficient samples were reduced to 20% of that of the control. JNCL patient fibroblasts and overexpression studies revealed a positive correlation with CLN3 expression and BMP levels. BMP, or LBPA (lysobisphosphatic acid), is found especially in the internal membranes of multivesicular late endosomes (Kobayashi et al. 1998), an organelle where CLN3 is also localized. LBPA is enriched in raft-like domains in the internal membranes of these organelles and involved in lipid and protein sorting in the endosomal/lysosomal system (Kobayashi and Hirabayashi 2000). Interestingly, Luiro and co-workers have reported defects in membrane trafficking in JNCL fibroblasts (Luiro et al. 2004). An LDL receptor-mediated degradative endocytosis experiment showed that by the endocytic time-course, LDL predominantly co-localized with the LBPA-labeled late endosomes in wild type cells, whereas LDL did not reach the late endosomes but was exclusively found in the early endosomes in CLN3-deficient cells. The missing co-localization of LDL and LBPA could result from the diminished LBPA content in JNCL cells. Together, these data implicate a role for CLN3 in the maintenance of the characteristics of the late endosomal membrane, important for lysosomal maturation and degradation of lipids and membrane-bound proteins. It would be interesting to see whether these potential new functions of CLN3 could be connected to the previously reported associations with cytoskeletal dysfunctions (Luiro et al. 2006).

The sequence of CLN8, an ER-Golgi–resident transmembrane protein associated with vLINCL and Northern epilepsy, was examined *in silico* and found to be homologous to a large protein family with a TLC domain (TRAM, Lag1, CLN8) (Winter and Ponting 2002). The members of this protein family are linked to the synthesis, transport, and sensing of lipids. CLN8 was suggested to have a possible role in ceramide synthesis and the transport of ceramide between the ER and Golgi. Indeed, liquid chromatographic and mass spectrometric analyses of two CLN8-deficient patients' cerebral samples revealed abnormal sphingo- and phospholipid levels (Hermansson et al. 2005). The analyzed patients were in different stages of the disease, and the lipid composition varied between them. The patient with the less advanced disease showed reduced levels of ceramide, galactosyl- and lactosylceramide, and a decrease of lipids from these sphingolipid classes containing long fatty acids.

Cathepsin D, involved in congenital and late infantile NCLs, has also been linked to lipid metabolism. Its proteolytic activity has been suggested to be induced by ceramide binding (Heinrich et al. 2000). CTSD has been reported to regulate ABCA1-mediated lipid efflux from macrophages and CHO cells (Haidar et al. 2006). CTSD inhibition led to over 70% reduction of apoA-I mediated lipid efflux

via reduced ABCA1 expression and mislocalization. Accumulation of glycsphingolipid and free cholesterol was detected in late endosomes/lysosomes.

2.4.5 INCL – clinical picture

Infantile NCL (INCL, Santavuori-Haltia disease, MIM 256730) is caused by mutations in the *CLN1* gene, resulting in the deficiency of palmitoyl protein thioesterase 1 (PPT1) (Jarvela 1991, Vesa et al. 1995). INCL was described by the Finnish pediatrician Pirkko Santavuori and co-workes in a study of 15 patients (Santavuori et al. 1973). The disease is enriched in the Finnish population, with an incidence of 1:20 000 and a carrier frequency of 1:70. Affected newborns are healthy and develop normally until the first symptoms, protracted head growth and muscular hypotonia, are seen around the age of six months. Magnetic resonance imaging shows hypointense thalami in T2-weighted images very early in the course of the disease, even before the first clinical signs (Santavuori et al. 2001). Sleep problems, irritability, visual failure, ataxia, and motor clumsiness develop, and during the second year of life the deterioration of motor and mental development is rapid (Williams et al. 2006). By the age of three, the EEG of the patients is isoelectric and they have lost active movements and social and visual contact with their environment. Death usually occurs around 10 years of age.

In addition to infantile NCL, mutations in the *CLN1* gene can also result in a disease with delayed onset. Mutations in the *CLN1* gene have been estimated to cause about 20% of NCL cases in the US, and only half of them presented in infants (Wisniewski et al. 2001). Some mutations are connected to the late infantile or juvenile disease types, which clinically resemble other NCLs with later onset (Kalviainen et al. 2007, Mitchison et al. 1998, Mole et al. 2001). Interestingly, three adult cases have also been described. Disease onset occurs in the third or fourth decade of life and its progression is slow. The first signs of neurodegeneration are depression, cognitive decline, impaired vision, and hallucinations, later resulting in dementia, ataxia, and motor weakness (Ramadan et al. 2007, van Diggelen et al. 2001).

2.4.6 Neuropathology of INCL

The fundamental neuropathological findings in INCL were described in parallel with the clinical findings in the early 1970's by the Finnish neuropathologist Matti Haltia (Haltia et al. 1973). Frontal brain biopsies from 1,5-year-old patients show progressive neuronal loss, severe astrocytic hyperplasia and hypertrophy, and macrophage infiltration in the cortex. All cell types contain autofluorescent storage granules. In older patients (3 years) the cortical neurons are almost totally lost and

macrophages dominate the cortex. Autopsy samples show striking generalized brain atrophy (**Figure 5**). Both the cerebral and cerebellar cortices are depleted of neurons, except for a few giant cells of Betz and occasional hippocampal CA1 pyramidal cells and Purkinje cells. Hypertrophic astrocytes with coarse processes form a rim in the cortices. Neurons in the basal ganglia and brainstem are less affected, and neurons in the spine and ganglia are well preserved despite significant storage. In all parts of the brain, a subtotal to total loss of myelin and fibrillary astrogliosis is detected.

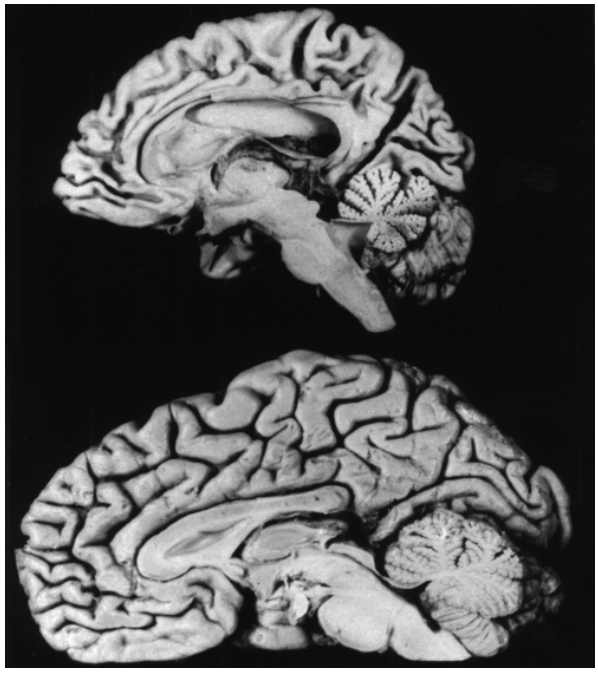

Figure 5. ***Sagittal sections of the brains of an INCL patient (above) and a normal control (below). The INCL brain is severely atrophied; especically the volume of the cerebral cortex is diminished. Figure courtesy of Prof. Juhani Rapola.***

2.4.7 Intracellular storage material in INCL

Autofluorescent lysosomal storage material with an ultrastructure of granular osmiophilic deposits (GROD) is characteristic to all diseases with mutations in *CLN1*, independent of the age of disease onset (**Figure 6**). The main protein

components in GRODs are saposins A and D, which seem to determine the storage ultrastructure (Tyynela et al. 1993). GRODs together with at least saposin D storage are also found in congenital NCL caused by the deficiency of cathepsin D (Siintola et al. 2006). Saposins are sphingolipid activator proteins derived from a common precursor polypeptide called prosaposin via proteolytic cleavage (discussed in Chapter 2.2.2). Saposin A is required for the lysosomal degradation of galactosylceramide (cerebroside) (Matsuda et al. 2001) and saposin D is involved in the degradation of ceramide (Azuma et al. 1994). Interestingly, cathepsin D is involved in the proteolysis of prosaposin (Hiraiwa et al. 1997). Prosaposin is widely expressed in various tissues, and in addition to serving as a precursor for mature saposins, it is secreted into various bodily fluids such as milk, bile, and semen (Hineno et al. 1991). In the brain, prosaposin functions as a potent neurotrophic factor (O'Brien et al. 1994). An alternative splicing of prosaposin is linked to brain development, the percentage of the longer mRNA isoform increasing during embryogenesis (Cohen et al. 2004). It is also linked to neuronal regeneration, as the expression pattern of the two mRNA forms present in the rat is changed after nerve injury (Chen et al. 2008, Hiraiwa et al. 2003).

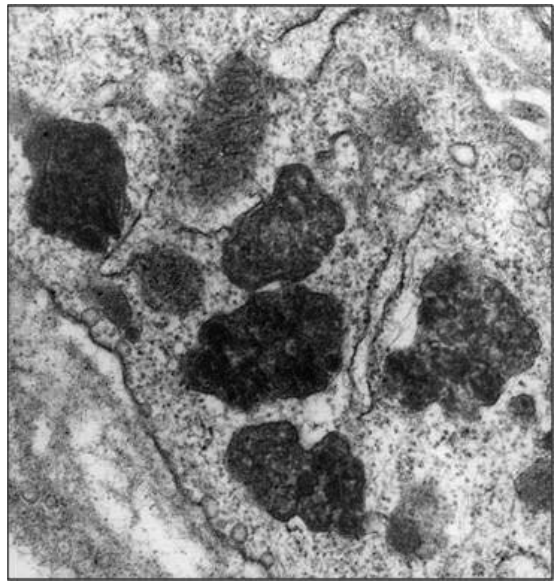

Figure 6. ***Electron micrograph of granular osmiophilic storage deposits (GRODs) in the cortex of an INCL patient (autopsy sample). Figure courtesy of Prof. Juhani Rapola.***

Saposins are highly hydrophobic proteins, and in addition to INCL, they tend to accumulate in many other lysosomal storage disorders as well, such as type A Niemann-Pick, Tay-Sachs, and Gaucher disease. However, in these diseases, saposins accumulate together with associated sphingolipids after primary defects in sphingolipid metabolism (Morimoto et al. 1990), while in INCL the protein-to-lipid ratio is ten times higher (>1). Saposins accumulate in minor amounts also in those forms of NCL where the major storage protein is subunit c of ATP synthase (Tyynela et al. 1995). However, subunit c does not accumulate in INCL, although in addition to other NCLs it is also stored in many other lysosomal storage disorders.

The cellular events leading to storage accumulation in INCL and its relation to the disease pathogenesis are not known. Direct interactions between saposins and PPT1 have not been shown to occur, and saposins do not possess S-acylation. The storage material can already be detected in the fetus during the first trimester of pregnancy (Rapola et al. 1990). However, in some cases, the first symptoms of neurodegeneration may appear as late as in the third decade of life. This speaks for the hypothesis that the storage itself is not the cause of neurodegeneration. Still, elucidating the events behind the storage accumulation may reveal important mechanisms of INCL pathogenesis. In fact, abnormal processing and localization of saposins, possibly linked to defects in endocytosis, were recently detected in INCL fibroblasts and Ppt1-deficient neurons (Ahtiainen et al. 2006). In INCL fibroblasts, the processing of prosaposin was found to be abnormal, and prosaposin was also hypersecreted. The amounts of saposins A and D in INCL fibroblasts were significantly increased, being nearly ten times higher than in the wild type cells. This is interesting in the light of a study characterizising the interactions of saposins with model lipid bilayers using atomic force microscopy (AMF) (Alattia et al. 2006). The authors report that saposin A forms stable and mechanically resistant aggregates at a protein concentration of 50μM, which is approximately ten times higher than normal physiological conditions. Perhaps the storage accumulation in INCL is due to increased concentrations of saposins A and D in the organelles of the endocytic pathway. Still, it is not known why the amounts of these two proteins are increased when at the same time the localization and concentrations of saposins B and C are normal in INCL cells.

2.4.8 *CLN1* gene and mutations

The *CLN1* gene (ceroid lipofuscinosis, neuronal) encoding for the PPT1 enzyme was identified by linkage analysis and positional cloning in chromosome 1p32 in 1995 (Jarvela 1991, Vesa et al. 1995). *CLN1* is conserved among species, having orthologues in vertebrates as well as in the fruit fly (*Drosophila melanogaster*),

roundworm (*Caenorhabtidis elegans*), and fission yeast (*Schizosaccharomyces pombe*), suggesting a fundamental cellular function for the gene (experimental models are discussed in Chapter 2.4.11) (Cho and Hofmann 2004, Korey and MacDonald 2003, Porter et al. 2005). In the human, *CLN1* is a 25-kb gene containing 9 exons and producing a single 2.5 kb mRNA. The gene defect behind the classical INCL found homozygously in most Finnish cases is a missense mutation (c.364A>T, Arg122Trp) leading to disrupted transport and inactivity of the PPT1 enzyme. Other common mutations in *CLN1* include two nonsense mutations (c.29T>A, Leu10X; c.451C>T, Arg151X) and a missense mutation (c.223A>T, Thr75Pro). To date, 45 disease-causing mutations have been described in the *CLN1* gene (NCL Resource database, www.ucl.ac.uk/ncl/cln1.shtml, S. Mole). Mutations are found throughout the gene, and most of them affect only individual families. No clear genotype-phenotype correlation has been observed. This may be due to small sample cohorts, although the possible roles of other underlying factors implicated in some other LSDs, such as modifier genes, remain to be studied.

2.4.9 The PPT1 protein

Structure and modifications

Palmitoyl protein thioesterase (PPT1) polypeptide contains 306 amino acids, including a 25-aa-long N-terminal signal sequence which is cleaved co-translationally after translocation into the endoplasmic reticulum. The crystal structure of bovine PPT1 (94% identical to human PPT1) has been determined by X-ray crystallography (Bellizzi et al. 2000). The enzyme possesses a classical α/β serine hydrolase structure composed of two major domains and a catalytic triad (Ser115, Asp233 and His289). In the center of the molecule lies a hydrophobic fatty acid binding groove (**Figure 7**). PPT1 has three asparagine-linked glycosylation sites at positions 197, 212, and 232. All of them are used and shown to affect the activity and/or transport of the enzyme (Bellizzi et al. 2000, Das et al. 2001).

Previous studies suggest PPT1 to exist in different biochemical environments, proposing possible additional modifications in PPT1. PPT1 was first described when investigating the palmitate cleavage of a model palmitoylated substrate, the H-Ras oncogene (Camp and Hofmann 1993). When different rat tissues were tested for depalmitoylating activity, the brain cytosolic fraction held the highest activity together with spleen, seminal vesicle, and testicular cytosolic fractions. However, 10 to 25% of the activity was always associated with membranes. It is not known if PPT1 is partly membrane-associated, or whether the activity is derived from another enzyme capable of depalmitoylating H-Ras. The only enzyme described to perform that function is cytosolic APT1, for which membrane association has not been

reported (Smotrys and Linder 2004). However, similar membrane association to PPT1 was detected when APT1 was purified (Duncan and Gilman 1998). In addition, overexpressed PPT1 has been found to partly associate with detergent-resistant microdomains (Goswami et al. 2005).

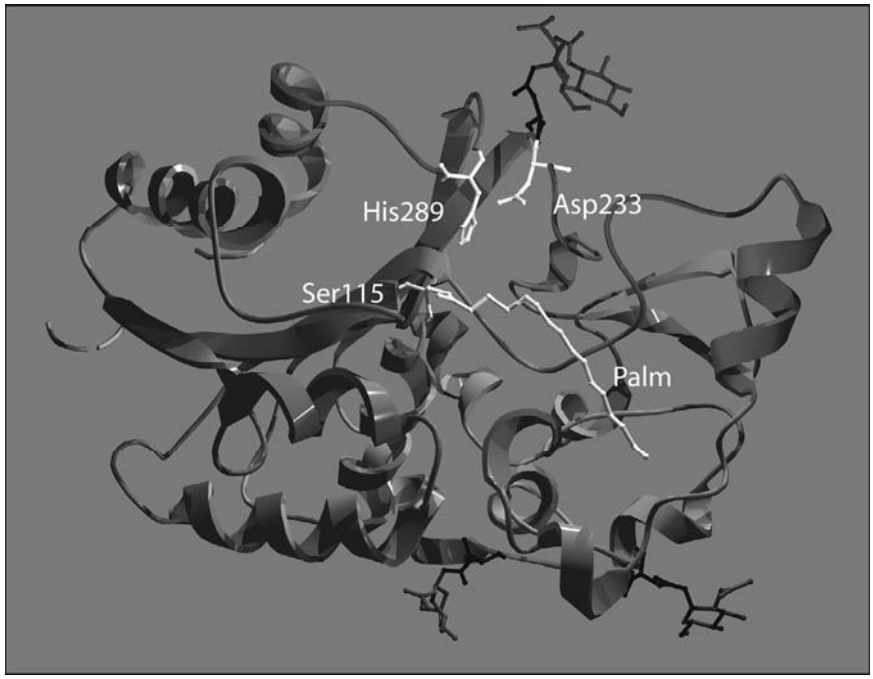

Figure 7. ***The molecular structure of PPT1. The three-dimensional structure of the native bovine palmitoyl protein thioesterase 1 (PPT1) complexed with palmitate (1EH5, Protein Data Bank, http://www.rcsb.org/pdb/). The polypeptide backbone structure is shown as α-helices, β-sheets, and coils. The three N-linked glycan structures (grey) on the surface of the molecule and the respective glycosylated asparagine amino acids (black) are shown as stick models. The amino acids of the catalytic triad are labeled and drawn as white stick models. PPT1's substrate, palmitate, binds in a hydrophobic groove on the surface of PPT1 and is shown here as complexed with Ser115. The image was made with DeepView / Swiss PdbViewer 3.7 (SP5) and rendered with POV-Ray.***

PPT1 also separated into two fractions later in the purification from bovine brain cytosol by Camp and co-workers. During the last step of purification with hydroxyapatite chromatography and increasing NaCl concentration, the enzyme activity was separated into two fractions. The first showed PPT1 with several other minor bands that did not correlate with enzyme activity, and the second as a homogenous band. The separation of the enzyme into two forms could not be explained, but protein phosphorylation was thought to be a possible reason for it.

A more recent proteomic study utilizing post-mortem human brain mannose 6-phophorylated samples showed as many as 32 different isoforms for PPT1 in 2-D gel analyses (Sleat et al. 2005). Each glycosylation form was separated into approximately 9 isoforms with different pI. Changes in the isoelectric point of a protein occur when the net charge of a protein is changed, for example after amino acid substitution, substrate binding or other interactions, or after post-translational modifications.

Function

PPT1 was originally characterized as a classic soluble lysosomal enzyme participating in the catabolism of S-acylated proteins. In human lymphoblasts, PPT1 removes thioester-linked fatty acids from cysteine-residues in proteins (Lu et al. 1996). The *in vivo* substrates of PPT1 are not known, but *in vitro* experiments have shown PPT1 to be able to remove palmitate and other fatty acids from peptides and proteins such as GAP-43 (growth-associated protein) and rhodopsin (Cho et al. 2000b). The pH scale for PPT1 activity is broad, the optimum being at 6.5 to 7.0. It is unusually high for a lysosomal enzyme and suggests functionality also outside of lysosomes.

PPT1 has a homolog, PPT2, which shares 26% identity with PPT1. The crystal structures of the two proteins are very similar (Calero et al. 2003) but their substrate specificity varies: PPT1 has a more restricted range of fatty acyl chain lengths (optimum at 14 to 18) whereas PPT2 also has thioesterase activity against lipids with less than 10 and more than 18 carbons. While PPT1 is able to depalmitoylate proteins, PPT2 cannot bind to fatty acids with bulky head groups. PPT2 is not known to be mutated in humans, but the Ppt2-deficient mouse model develops an NCL-like phenotype with neurovisceral features (Gupta et al. 2003).

Localization

PPT1 is expressed abundantly in most tissues. In the mouse brain, the expression of PPT1 starts as early as the eighth embryonic day and it is developmentally regulated in the rat and mouse brain (Isosomppi et al. 1999, Suopanki et al. 1999a). This is also the case in humans, as PPT1 expression was shown to increase during

corticogenesis in the human embryonic brain by *in situ* hybridization and immunohistochemistry (Heinonen et al. 2000b). Studies on neuron cultures showed that PPT1 expression increases during neuron maturation and precedes the expression of synaptic markers (Ahtiainen et al. 2003).

In non-neuronal cells, PPT1 is lysosomally targeted and utilizes the mannose 6-phosphate receptor-mediated pathway (Hellsten et al. 1996, Verkruyse and Hofmann 1996, Verkruyse et al. 1997). In murine primary neuron cultures, SFV-mediated PPT1 showed colocalization with synaptic vesicle protein SV2 while no visible colocalization with lysosomal LAMP1 was detected in immunofluorescence studies. Endogenous PPT1 showed similar distribution in mouse brain fractionation studies (Lehtovirta et al. 2001). This finding has been replicated by immunofluorescence studies with endogenous PPT1 (Ahtiainen et al. 2003, Suopanki et al. 2002), although negative findings also exist (Virmani et al. 2005). After excitotoxicity induced by kainic acid in adult rats, the expression of PPT1 was increased and its colocalization with the synaptic membrane marker NMDAR2B was enhanced, suggesting a role for PPT1 in synaptic plasticity (Suopanki et al. 2002).

2.4.10 PPT1 and membrane lipids

The phospholipid content of post-mortem brain samples of INCL patients has been analyzed with chromatography and mass spectrometric methods (Kakela et al. 2003). Over 60% of the normal phospholipid content was lost due to neuron loss and demyelination, and the remaining proportions of phospholipids were altered with an increase in phosphatidylcholine and decrease in phosphatidylserine and phosphatidylethanolamine levels. Lysobisphosphatic acid (LBPA or BMP) content was nearly 100 times higher in the INCL brain than in controls and LBPA was found to be rich in polyunsaturated fatty acids. The authors discussed that high LBPA content could be derived from increased autophagocytosis and lysosomal proliferation. However, as seen in CLN8 patients (Hermansson et al. 2005), the brain lipid content varies greatly when the disease progresses and further studies are needed to detect early changes in brain lipids. The complex architecture of the brain may also contribute to variable findings, since the lipid content varies between different brain regions.

In another study, the overexpression of PPT1 led to a decrease in the ceramide content of lipid rafts while it was increased by the overexpression of neutral sphingomyelinase (Goswami et al. 2005). The results are in line with the function of the proteins; sphingomyelinases catalyse the degradation of sphingomyelin to ceramide and phosphocholine, and PPT1 deficiency leads to abnormal processing of saposin D, which is involved in ceramide degradation. Interestingly, a fraction of the

overexpressed proteins was also shown to localize to rafts, suggesting a possible function for PPT1 in the plasma membrane.

2.4.11 Simple experimental models for INCL

An efficient way to study the human disease pathogenesis is provided by simple experimental disease models, such as yeast, the fruit fly, and a nematode worm. The *CLN1* gene is conserved in eukaryotes, making the development of INCL disease models possible. While in budding yeast (*S. cerevisiae*) PPT1 does not have an ortholog, in fission yeast (*S. pombe*) such a gene exists, possessing 31% identity with the human PPT1. The Ppt1-encoding gene is fused in frame with the Dolpp1 gene (ortholog for *DOLPP1*, dolichol pyrophosphate phosphatase-1). This fusion gene *pdf1* encodes the precursor protein Pdf1p, which is cleaved into Ppt1p and Dolpp1p. Dolpp1p deficiency is lethal while Ppt1p-deficient cells are viable. However, cells containing no functional copy of Ppt1p were abnormally sensitive to vanadate and elevated extracellular pH – phenotypes linked to dysfunction in vacuolar acidification and vacuolar protein sorting. Vacuoles in yeast are equivalent to lysosomes in mammals. Human PPT1 was able to complement these phenotypes, suggesting an evolutionarily conserved function for PPT1 (Cho and Hofmann 2004).

C. elegans, a nematode worm, has a completely mapped cell lineage and nervous system and is therefore often used in neurobiological studies. *C. elegans* has an ortholog gene, *ppt-1,* for the human *CLN1,* with 54% identity and 75% similarity. After *ppt-1* knockout mutation, the overall phenotype of the worms was mild, and no alterations or defects in life span, body size, or locomotion were detected. Storage material also was not detected. Residual PPT1-like activity was detected in the knockout worms, suggesting the presence of a compensatory enzyme. The most striking finding concerned the myocytic and neuronal mitochondria, which were structurally abnormal, showing membrane whorling and abnormal cristae. The overall number and total area of mitochondria were also increased. The defect was the strongest at the first day of adulthood and decreased during the course of life. Thus, the authors suggested that ppt-1/PPT1 could be involved in the regulation or biogenesis of this organelle. (Porter et al. 2005) Mitochondrial dysfunction has also been linked to NCLs previously (see Chapter 2.4.3).

Drosophila melanogaster, a fruit fly, has an ortholog of human PPT1 (55% identical and 72% similar), but the overall PPT1 activity in the fly, as well as in *C. elegans*, is significantly lower than in mammals (Glaser et al. 2003). Regardless, Ppt1 deficiency in the fly leads to abnormal storage material accumulation and reduced lifespan, although no obvious neurodegeneration is seen (Hickey et al. 2006). The overexpression of Ppt1 results in increased apoptosis in the developing visual

system (rough eye) (Korey and MacDonald 2003). Drosophila is a model commonly used for modifier gene screening, and this procedure was used to elucidate the function of Ppt1 as well. Ppt1-overexpressing stock was crossed with nearly 2000 other genetically modified stocks and the progeny were screened for either enhanced or suppressed eye degeneration phenotype (Buff et al. 2007). The screen revealed ten enhancer and ten suppressor genes for Ppt1-induced degeneration. Modifier genes were involved in endosomal trafficking and lipid metabolism, ubiquination, cell adhesion, and signalling pathways. Some of the most interesting enhancers were *endophilin A* and *blue cheese* (Bchs), involved in synaptic vesicle recycling and endo-lysosomal maturation in neurons. Two genes participating in lipid metabolism, phosphatidylserine decarboxylase and ATP-binding cassette homolog, were also enhancers of Ppt1. Ppt1 suppressors included *Hsc70-3*, a fly homolog of BiP, an ER-resident chaperone. Two suppressors involved in synaptic function were synaptotagmin and stoned A, an adaptor protein involved in the recycling of synaptotagmin. Synaptotagmin is a Ca^{2+}-sensor for synaptic vesicle fusion and neurotransmitter release at the active zone. Overall, these findings support the suggested connection between INCL and early defects in the function of synapses. The results await replication in higher eukaryotic systems, since although the exocytic proteins in presynaptic terminals are conserved between insects and the human, the same is not true for the endocytic proteins (Yanay et al. 2008). Thus the synapse maturation in the human is likely to differ from that in the fly. Additionally, recent links between lipid metabolism and NCL disorders may not be optimally studied in Drosophila due to differences in lipid metabolic routes of mammals and Drosophila, as evidenced by the inability of the fly to synthesize cholesterol (Gilbert et al. 2002).

2.4.12 INCL mouse models

Although the above-mentioned simple experimental models have their own advantages for studying human diseases, they do not provide enough information about the subtle neurobiological changes and cascades behind NCL disease pathogenesis. There are also some obvious limitations in studying the neurodegeneration in patients, although for example imaging techniques have provided new information about the early events in neuronal dysfunction. In order to understand the functional consequences of the disease mutation, and to evaluate possible treatments, it is necessary to model the diseases in mammals.

The mouse (*Mus musculus*) is the most important disease model in molecular medicine for many reasons: the mouse genome is fully sequenced and techniques for genetic engineering are refined, mice reproduce fast and are relatively easy to

maintain, and most importantly, mice share conserved physiological pathways and a similar genome with the human (Mouse Genome Sequencing et al. 2002). Mouse Ppt1 protein is 84.6% identical and 96.3% similar to the human PPT1 (Salonen et al. 1998). For INCL research, two different knock-out mouse models have been developed. The *Ppt1-/-* mouse by Gupta and co-workers is made by inserting a stop codon mutation into exon 9 (Gupta et al. 2001), and the $Ppt1^{\Delta ex4}$ mouse by Jalanko and co-workers is made by a complete deletion of exon 4 (Jalanko et al. 2005). Both models lack Ppt1 enzyme activity and develop an INCL-like phenotype. Intracellular storage material (GROD) accumulates and mice display early-onset progressive neurodegeneration and a shortened lifespan. $Ppt1^{\Delta ex4}$ mice show loss of vision at the age of 2 months, seizures from 4 months onward, paralysis of the hind limbs at 5 months, followed by death a few weeks later. Ppt1-deficient mice have provided crucial information about INCL disease pathogenesis (discussed in more detail below). In addition, they are utilized in biochemical and cell biological research and offer the possibility to evaluate the efficacy of treatments before proceeding into clinical trials with patients.

2.4.13 Treatment strategies for INCL

Three therapeutic trials for the treatment of Ppt1-deficient mice utilizing adeno-associated virus 2 (AAV2) -mediated PPT1 gene therapy have been reported (Griffey et al. 2004, Griffey et al. 2005, Griffey et al. 2006). The idea behind the virus-mediated gene therapy in lysosomal disorders is based on the trafficking of soluble lysosomal enzymes: when infected cells overproduce the missing lysosomal protein, they also secrete it. Neighboring cells are then able to endocytose the protein by utilizing the CI-MPR and to thus complement their deficiency. AAV-mediated gene therapy trials have been successful with many different lysosomal enzymes. A trial to treat LINCL (the deficiency of TPP1) showed long-lasting correction of enzyme activity and efficient enzyme diffusion throughout the mouse brain and positive results were also obtained in nonhuman primates (Hackett et al. 2005, Passini et al. 2006, Sondhi et al. 2005, Sondhi et al. 2007). Consequently, human trials have been initiated to treat LINCL (Crystal et al. 2004). Although Ppt1-deficient mice also showed some improvement in brain histology and behaviour, there was no correspondence in seizure activity or life span. This was due to a limited diffusion of the enzyme in the mouse brain, proposing that Ppt1-trafficking may not follow the classic route of lysosomal enzymes in neurons. Recent publications have described novel routes for lysosomal transport, mediated for example by sortilin and LIMP-2 (discussed in Chapter 2.2.3). It will be interesting to analyze whether alternative trafficking routes also exist for PPT1.

2.4.14 Suggested mechanisms behind neurodegeneration in INCL

Neurodegeneration in INCL has been proposed to be caused by several underlying mechanisms, the increased vulnerability of neurons to apoptosis being one of them. Apoptotic mechanisms are involved in INCL as increased apoptosis has been detected in the brain biopsies of INCL patients (Riikonen et al. 2000). Studies utilizing PPT1 overexpression and inhibition in LA-N-5 neuroblastoma cells have suggested PPT1 to act as an anti-apoptotic protein (Cho et al. 2000a, Cho and Dawson 2000). Cancer studies support this finding, as increased PPT1 expression is seen in colorectal cancer metastasis (Tsukamoto et al. 2006) and inhibition of PPT1 kills cultured tumor cells (Dawson et al. 2002). A recent large-scale proteomic approach revealed an interaction between PPT1 and the c-MYC oncogene, linking PPT1 more tightly to cell cycle regulation (Koch et al. 2007, Trumpp et al. 2001). However, the function of this interaction was not discussed and its location is unknown, as PPT1 is intravesicular and c-MYC resides in the cytosol and nucleus. Increased expression of other NCL proteins (CTSD, TPP1 and CLN3) in cancer has also been reported (Junaid et al. 2000, Rylova et al. 2002).

Increased apoptosis in the INCL brain has been proposed to result from ER stress leading to the unfolded protein response and apoptosis via activation of caspase-4 and caspase-3 (Kim et al. 2006b, Zhang et al. 2006). ER stress has also been suggested to increase the amount of reactive oxygen species that destabilize the mitochondrial membrane and activate caspase-9-mediated apoptosis (Kim et al. 2006c). However, ER and oxidative stresses were later shown to also be common mediators of apoptosis in many other lysosomal storage disorders with or without neuronal manifestations, and thus not specific for PPT1 deficiency (Wei et al. 2008).

Neuronal apoptosis is followed by the removal of the cell corpses by phagocytes (activated microglia, astroglia, and macrophages). Growing evidence suggests that although microglial activation is crucial to neuron survival and host defence, overactivation can lead to increased neurotoxicity and progressive cell death (Block et al. 2007). Microgliosis is also evident in the INCL brain. Increased levels of lysophosphatidylcholine, a chemoattractant for phagocytes, together with imperfect phagocytosis by the Ppt1-deficient phagocytes, were suggested to contribute to microgliosis and progressive neuronal death in the Ppt1-deficient mouse brain (Zhang et al. 2007).

The initiating steps in the disease course remained unknown until recent histopathological data from sheep and mouse models. Examination of mouse brains that model various NCL subtypes has pointed out that certain neuron populations are more vulnerable to the effects of the disease than others and that there is a special sequence of events that precedes the massive cortical neuronal death (Cooper et al.

2006). In the Ppt1-deficient mice, it is the thalamus that shows the first signs of neuron loss, together with localized activation of astrocytes and microglia (Kielar et al. 2007). The first affected nucleus in the thalamus is the lateral dorsal geniculate nucleus (LGNd) within the visual system, followed by the loss of neurons in the auditory and somatosensory nuclei. Cortical neuron loss appears only months after the loss of thalamic relay neurons (Bible et al. 2004) and is suggested to be the consequence of reduced afferent signalling from the thalamus. The affected neuronal populations have been identified to some extent and several studies indicate that the inhibitory GABAergic interneurons are severely affected in the Ppt1-deficient mice (Jalanko et al. 2005) as well as in all NCL mouse models studied (Cooper et al. 2006).

The reason for thalamocortical neuron loss is still elusive, but astrocytes have been suggested to play an important role in the pathogenesis. The distribution of astrocytosis predicts the subsequent distribution of neuron loss in many NCL mouse models and suggests a possible role for neuron-glia interactions in the course of disease (Cooper et al. 2006). Whether the principal glial activation is protective or harmful to neurons is not known, but as already mentioned, microglial overactivation potentially promotes neuronal death. Astrocytes are important in the maintenance of the synapse, and evidence of synaptic defects with a reduced pool of synaptic vesicles has been reported in Ppt1-deficient mice (Virmani et al. 2005). However, thorough electrophysiological recordings in acute hippocampal slices of the 1-month-old Ppt1$^{\Delta ex4}$ mice showed no abnormalities, suggesting normal synaptic function in these mice (Ahtiainen et al. 2007). This study further revealed that neuronal cholesterol metabolism, also highly interconnected with astrocytes and discussed in more detail in Chapter 2.3, show fundamental changes in the Ppt1-defient neurons (Ahtiainen et al. 2007). Gene expression-profiling studies on cultured neurons showed upregulation of cholesterol biosynthesis. Functional experiments certified the gene expression data; the rate of sterol biosynthesis and conversion of sterol precursors into cholesterol were upregulated, but the amounts of intracellular cholesterol and precursors were constant or diminished, respectively. Maintenance of the correct lipid composition in neuronal membranes is essential for the correct function of the cell and any disruption in it is more than likely to disturb neuronal development or aggravate, if not cause, the neuronal death.

2.4.15 vLINCL$_{Fin}$ – clinical findings

Mutations in the *CLN5* gene result in the Finnish variant late infantile NCL (MIM 256731) (Santavuori et al. 1982), named so because it is enriched and was described in Finland, and it is distinctive both clinically and neuropathologically from the classic LINCL caused by mutations in the *CLN2* gene. The first clinical signs are

observed between the ages of 4 to 6 years, first with attention deficits and motor clumsiness, followed by learning problems, visual impairment, and mental decline. Epileptic seizures, ataxia, and myoclonic jerks are important features of the disease and manifest between 7 to 10 years of age. Magnetic resonance imaging shows early cerebellar atrophy, and T2-weighted images show decreased signal intensity of the thalami (Autti et al. 1992). Progressive optic atrophy and macular dystrophy lead to blindness before the age of ten, and patients survive until 14 to 32 years of age (Santavuori et al. 2000).

2.4.16 Neuropathology of vLINCL$_{Fin}$

Neuropathologically vLINCL$_{Fin}$ is characterized by severe cerebral and extreme cerebellar atrophy. The cerebellar Purkinje and granular cells are almost totally obliterated, while most subcortical structures show relatively modest neuronal loss. The cerebral neuron loss is particularly evident in layers III and V, and there is severe cortical astrocytosis and loss of myelin in the white matter. The ultrastructure of the intraneuronal storage bodies possesses both rectilinear complexes and curvilinear profiles (**Figure 8**). Some cells also show fingerprint patterns. The main storage protein component is subunit c of ATP synthase, and minor amounts of SAPs A and D can also be detected (Tyynela et al. 1997).

2.4.17 CLN5 gene and mutations

The *CLN5* gene, identified in 1998, is located on chromosome 13q22 and has four exons. It is expressed widely in different tissues (Savukoski et al. 1998). The *CLN5* gene is conserved only in vertebrates (Siintola et al. 2006). Immunohistochemical studies, together with *in situ* hybridization, showed that the expression of *CLN5* in the human brain begins at the early developmental stage and increases during corticogenesis (Heinonen et al. 2000b). Expression analyses in the mouse showed similar results of increasing expression during brain development. In the adult mouse brain, *Cln5* expression is most intense in the cerebellar Purkinje cells, cerebral cortex, and the principal cell layers of the hippocampus (Holmberg et al. 2004). This is well in line with the neuropathological findings in humans. Over 90% of the Finnish CLN5 patients carry the major mutation (CLN5$_{FinMajor}$, c.1175delAT, Y392X), which is predicted to lead to a 16-aa truncation of the CLN5 polypeptide (Savukoski et al. 1998). To date, mutations in all four exons have also been found in families from other European countries and Latin America, and the present number of reported mutations in the CLN5 gene is 13 (NCL resource database, http://www.ucl.ac.uk/ncl/cln5.shtml).

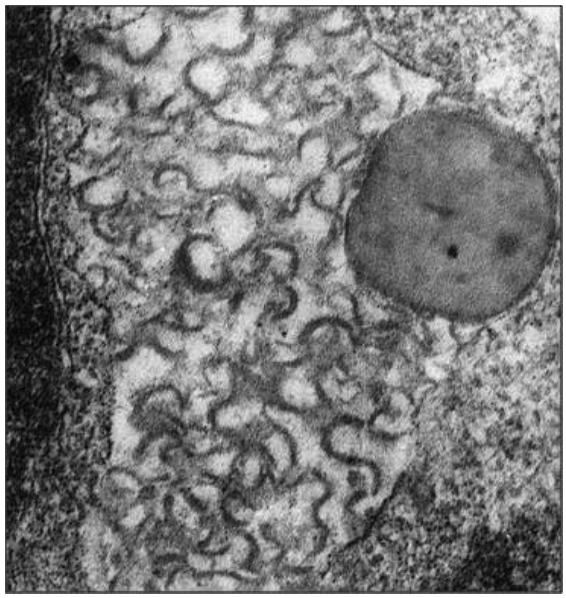

Figure 8. ***Lysosomal storage material in a vLINCL$_{Fin}$ patient's tissue forms curvilinear profiles. Figure courtesy of Prof. Juhani Rapola.***

2.4.18 The CLN5 protein

The CLN5 protein shows no homology to other known proteins and its function is still unknown. The human *CLN5* coding sequence contains four possible ATG initiation sites, and it was initially suggested that the most 5' upstream site was the functional one, producing a 407-aa polypeptide with two transmembrane domains. However, subsequent protein expression studies revealed expression of a highly glycosylated protein migrating as a 60-kDa band. After deglycosylation with PNGase F, the size decreased down to 38 kDa, smaller than the theoretical size of a polypeptide translated from the first methionine. These results together with an alignment with the mouse *Cln5* gene suggest that the fourth methionine is the major initiation site, leading to an expression of a soluble lysosomal glycoprotein. Lysosomal trafficking was shown to be blocked when the CLN5 polypeptide carried the major Finnish mutation (Holmberg et al. 2004, Isosomppi et al. 2002). Supporting evidence for the solubility and lysosomal localization has been derived from proteomic studies where CLN5 has been found to possess a classic lysosomal mannose 6-phosphate targeting motif (Sleat et al. 2005, Sleat et al. 2007). Only one ATG initiation site is present in the CLN5 gene in

mice, dogs, cattle, and sheep, and recent alignments with the human sequence suggest that it is the third, rather than the fourth, human initiation site that is evolutionary conserved (Frugier et al. 2008). This start codon would result in a soluble protein of 358 amino acids. It has been suggested that the unusually long 5' UTR region in the originally reported human *CLN5* gene could actually represent the pre-mRNA form of *CLN5*, containing the 5' end intron which could participate in the regulation of tissue-specific *CLN5* gene expression (Isosomppi 2003). This type of regulation has been demonstrated to occur for example in the RNA-editing ADAR enzymes (Lykke-Andersen et al. 2007). In the future, it would be very interesting to characterize the human CLN5 protein in more detail and to investigate the role of the four possible initiation sites in the expression of CLN5 in different tissues in order to better understand its function.

2.4.19 Animal models for vLINCL$_{Fin}$

A Cln5-deficient mouse model has been developed by a targeted deletion of exon 3 of the mouse *Cln5* gene (Kopra et al. 2004). The mouse model shows the accumulation of autofluorescent material and loss of vision, but no significant brain atrophy. Downregulation of genes coding for the myelin components was detected, mirroring the loss of myelin detected in the vLINCL$_{Fin}$ patients. Loss of GABAergic interneurons was detected in several brain areas. Naturally occurring mutations have recently been found also in the canine, bovine, and ovine CLN5 genes (Frugier et al. 2008, Houweling et al. 2006, Melville et al. 2005a). Ovine CLN5 in Borderdale sheep resembles the human disease with respect to the clinical and pathological courses. One of the first symptoms in affected sheep is blindness, accompanied by a tendency to walk in circles (Jolly et al. 2002a). Their gyrencephalic brains, together with their size and human-like physiology, make sheep a valuable model for future therapeutic interventions.

3 AIMS OF THE STUDY

Prior to this study, PPT1 had been suggested to have a distinct extralysosomal role in neurons. The function of CLN5 was totally elusive. This study was initiated to describe protein interaction partners for PPT1 and CLN5 in order to elucidate their function *in vivo* and the cellular mechanisms involved in the pathogenesis of INCL and vLINCL$_{Fin}$. The aims addressed in this study were the following:

- To study the differences in PPT1 processing in neurons and non-neuronal cells, and to compare the trafficking of PPT1 to the classic lysosomal enzyme AGA

- To find interaction partners for PPT1 and CLN5

- To study the effects of these interactions on the localization and/or processing of the interaction partners in wild type and in PPT1- and CLN5-deficient cells

4 MATERIALS AND METHODS

The materials and methods used in this study are described in the original publications.

Table 2. ***Materials and methods used in this study***

Material or Method	Original publication
Adenovirus -mediated gene expression	I, II
Antibody internalization assay	I
Antibody production and purification	I
Apolipoprotein A-I internalization assay	II
Assay for PPT1 activity	I
Cell fractionation	I
Cell surface biotinylation	II
Cloning of cDNAs and other plasmid constructs	I, II, III
Confocal microscopy	I, II, III
Glycosylation analyses	I
Dissection and culturing of primary neurons and glial cells	I, II
DNA extraction	I, II, III
Immunohistochemistry	II, III
Immunoprecipitation	I
In vitro- binding assays with GFP- and GST-fusion proteins	I, II, III
Lipid analysis from mouse serum	II
Metabolic labeling	I
Mouse brain tissue preparation	II
Primary fibroblast cultures	II
Prevention of palmitoylation with 2-bromopalmitate	II
Protein detection by direct and indirect immunofluorescence	I, II, III
Protein detection by silver staining	II
Protein detection by Western analysis	I, II, III
Protein identification by mass spectrometry	II
Protein production in stabile CHO-cells	I
Protein production in E.coli	I, II, III
Protein purification by chromatographic methods	II
Quantification of immunofluorescence images	II
Semliki Forest virus –mediated gene expression	I
Surface plasmon resonance analysis (Biacore)	I, II
TIRF microscopy	II
Transient transfections	I, III

5 RESULTS AND DISCUSSION

5.1 Evidence of a neuron-specific pattern for PPT1 (I)

Preceding this study, the intracellular localization of PPT1 was shown to be lysosomal in non-neuronal cells (Hellsten et al. 1996, Verkruyse and Hofmann 1996). In neurons, however, the endogenous and overexpressed PPT1 was enriched in synaptosomes and synaptic vesicles rather than in lysosomes (Lehtovirta et al. 2001). Later it was demonstrated that PPT1 colocalizes with presynaptic markers in the axonal varicosities and that its activity increases during neuronal maturation (Ahtiainen et al. 2003). The expression of PPT1 in the CNS had also been shown to be developmentally regulated and to be concurrent with or slightly preceding synaptogenesis (Heinonen et al. 2000a, Isosomppi et al. 1999, Suopanki et al. 1999b). Since the most prominent result of PPT1 deficiency in INCL patients is the massive neuronal death and previous studies had suggested an extralysosomal function for PPT1 in neurons, it was meaningful to compare PPT1 expressed in neurons and in non-neuronal cells in order to reveal any differences in its processing. It was also of interest to compare the intracellular trafficking of PPT1 with another well characterized lysosomal enzyme to reveal possible differences in their localization.

5.1.1 PPT1 is differentially modified in neurons vs. non-neuronal cells

The comparison of neuronal and non-neuronal PPT1 was approached by overexpressing adenovirus-mediated PPT1 in mouse primary fibroblasts and neurons. Western blot analysis revealed a difference in the glycosylation pattern of PPT1 between the two cell types, and also an apparent difference in the migration of the glycosylated forms. To deglycosylate the proteins, samples were treated with PNGaseF, an amidase that cleaves between the innermost GlcNAc and asparagine residues, removing the N-linked oligosaccharides from the polypeptide chain. After the treatment, PPT1 separated into two bands in fibroblasts while only one band was detected in neurons. This neuronal band migrated in between the two bands from fibroblasts (I, Figure 3). The specific nature of this difference is unknown, but because the same cDNA was used to express PPT1 in both cell types, it is likely that the shift is due to difference in the post-translational modifications (PTM). So far, N-glycosylation is the only PTM described in PPT1. It is possible that in different cells, PPT1 is proteolytically differently trimmed, which could explain the small shift in size between fibroblast and neuronal PPT1. The two PPT1 bands in

fibroblasts after deglycosylation represent two forms of PPT1, of which only one is seen in neurons. Database searches suggest phosphorylation and S-palmitoylation as possible modifications for PPT1, while prenylation, sulfination, myristoylation, and GPI-anchoring are unlikely. Phosphorylation increases the negative charge of the protein, but it also increases its mass. Therefore, it could cause the protein to migrate differently in the gel, or does not necessarily affect the mobility of a protein at all. The samples were boiled and treated with β-mercaptoethanol to reduce protein disulfide bonds. This treatment has been reported to also reduce the thioester bonds in a concentration-dependent manner (Hausmann et al. 1998, Schmidt et al. 1988). In our experiment, the concentration of β-mercaptoethanol was 1.4 M, and similar concentrations have been reported to reduce 90% of S-palmitoylation. However, this aspect was not specifically studied here, and it thus remains for further studies to examine the nature of the modification. This experiment is the first to show structural differences in PPT1 between neurons and non-neuronal cells, and supports the previous evidence for the neuron-specific function of PPT1. One example of a protein with neuron-specific processing is cholecystokinin, a hormone released from the gut but also from cerebral and peripheral neurons (Rehfeld et al. 2003). Different prohormone convertases act on procholecystokinin in different tissues, but differences in the cell cycle and granule maturation also affect the deviating cholecystokinin patterns in the CNS and gut. This may also be true for PPT1 – membrane maturation and trafficking are likely to be different in fibroblasts and neurons, but cell-specific enzymes modifying PPT1 are possible as well.

5.1.2 Transport of PPT1 differs from that of the classic lysosomal enzyme AGA

PPT1 has been shown to undergo mannose 6-phosphorylation in non-neuronal cells (Hellsten et al. 1996, Verkruyse and Hofmann 1996). In neurons, this modification has not been studied. Gene therapy trials suggest that the transport of PPT1 in the CNS is different from traditional lysosomal enzymes such as aspartylglucosaminidase (AGA). The virus-mediated PPT1 enzyme does not diffuse as effectively in the mouse brain as AGA and other mannose 6-phophorylated enzymes (Griffey et al. 2006, Sondhi et al. 2005, Virta et al. 2006). Furthermore, our initial experiments suggested a very inefficient uptake of PPT1 from conditioned media into different cell cultures (data not shown). We compared the trafficking of PPT1 and AGA in mouse fibroblasts and neurons by overexpressing the adenovirus-mediated proteins and detecting them with specific antibodies using an antibody internalization assay rather than the traditional immunodetection, where antibody labelling is done after fixation of the cells. This method allowed us to detect PPT1 and AGA recycled back to the interior of the cell from the plasma membrane. Only a

small fraction of PPT1 was detected in LAMP-1 positive vesicles in fibroblasts (I, Figure 3C), whereas most of the AGA colocalized with lysosomal LAMP-1 (data not shown). Also, PPT1 positive vesicles were close to the plasma membrane and cell projections in neurons, distinctive from the perinuclear distribution of AGA.

These data, together with the above data about the possible different neuronal modifications of PPT1, strengthen the hypothesis of PPT1 utilizing another trafficking route in addition to the MPR-regulated pathway that is used by AGA and the majority of soluble lysosomal enzymes. Due to the severity of the INCL disease, PPT1 was originally anticipated to be indispensable to very basic neuronal functions, such as synaptic transmission or recovery from excitotoxic events. However, studies conducted by Ahtiainen and co-workers revealed an improved recovery of Ppt1-deficient neurons after glutamate-induced stress, and no effects on their electrophysiological properties (Ahtiainen et al. 2007). Compensatory mechanisms may play a role in this, as another study showed a progressively reduced number of synaptic vesicles (SVs) in the readily releasable pool in Ppt1-deficient neurons, but again no alterations in the active electrophysiological properties (Virmani et al. 2005). Based on the cellular localization, the vesicle pool of endocytosed PPT1 demonstrated here could represent early/recycling endosomes. Keeping in mind the reported SV localization of PPT1 in neurons, it has been speculated that the biogenesis of SVs could start via two overlapping routes: from the plasma membrane in an AP2-dependent pathway, and from endosomes in an AP3-dependent pathway (Bonanomi et al. 2006). In the future, it would be interesting to examine the trafficking of PPT1 in neurons mimicking I-cell disease to specify the importance of mannose 6-phosphorylation to its neuronal trafficking. Examining the role of the two adaptor proteins in the formation of SVs in the absence of PPT1, as well as their role in the localization of PPT1 to SVs, would be informative. The possible participation of PPT1 in other trafficking routes described for lysosomal proteins, especially those involving sortilin, could in part explain the unsatisfactory results from the gene therapy trials.

5.2 Protein interactions of PPT1 (I- II)

5.2.1 PPT1 activity resides in a high molecular weight complex (I)

In order to understand the putative diverse functions of PPT1 *in vivo*, it was of greatest interest to us to examine its interaction partners. As discussed in Chapter 2.4.9, brain-derived PPT1 has been observed separating into various types of fractions during previous purification procedures (Camp and Hofmann 1993). Thus, we initiated our analyses by investigating whether PPT1 resides in a monomeric

form intracellularly. We utilized the lysosome-containing fraction of the PC12 cells and separated the proteins according to their size by size-exclusion chromatography. We then analyzed the enzyme activities of endogenous PPT1 and AGA in these fractions. The lysosome-containing fraction (10 000 g pellet) also contains mitochondria and other dense membrane fractions. AGA activity eluted in three sequential fractions correlating to its native homodimeric size, whereas PPT1 activity was eluted in two major peaks, the first representing a molecular weight of over 100 kDa, and the second peak of approximately 40 kDa (I, Figure 4). The latter is likely to represent the monomeric PPT1, but the first suggests that the majority of PPT1 activity resides in a larger protein complex. Further analyses using a GFP-PPT1 pull down assay suggested that PPT1 forms complexes where it interacts with itself either directly or through other molecules (I, Figure 5A). The nature of the complex was not analyzed in this study, but in the following analyses a protein complex interacting with PPT1 was identified.

5.2.2 PPT1 interacts with F_1-ATP synthase (I, II)

To obtain PPT1 for antibody production and protein interaction experiments, we utilized stable CHO cells producing PPT1, and purified PPT1 from the medium with hydrophobic interaction and size-exclusion chromatographic methods. A 50-kDa protein repeatedly co-purified with PPT1. As co-secretion may be a sign of true interaction, we proceeded to attempt to identify this protein. After SDS-PAGE and Coomassie staining, the band was subjected to mass spectrometric analysis. The protein turned out to be the β-subunit of mitochondrial F_1-ATP synthase (II, Supplementary material). This finding was interesting due to the link to subunit c of the mitochondrial ATP synthase found in other NCLs. However, at the same time it was also inconsistent with the reported localizations of these two proteins. To analyze whether a true interaction existed between PPT1 and the β-subunit, we utilized purified proteins and performed the surface plasmon resonance assay. The result was negative between PPT1 and the β-subunit. However, when the whole F_1-complex was utilized, a weak but clear concentration-dependent interaction between PPT1 and the F_1-complex was detected (II, Figure 1). We further tested the interaction using the GST-PPT1 pull down assay, and both α- and β-subunits of the F_1-complex showed interaction with PPT1 (II, Figure 2). Originally, no other F_1-subunits except β were identified as purifying with PPT1. It is possible that the interaction indeed occurs between the β-subunit and PPT1, but that the correct conformation of the β-subunit is required. In the F_1-complex, three β-subunits and three α-subunits form the catalytic unit and the ADP/ATP-binding sites are located in the β-subunits. What could be the reason for the interaction between the F_1-complex and PPT1? Although F_o/F_1-subunits are not reported to be palmitoylated,

the palmitoylation site prediction program CSS-Palm (Zhou et al. 2006) predicts the following putative palmitoylation sites in them: F_o-subunit S – two sites in the mitochondrial import sequence and one in the mature protein; F_o-subunit C – one site in the mitochondrial import sequence; F_1-subunit gamma – one site in the mature protein. These sites could be targets for the depalmitoylation function of PPT1, and could have the potential to affect the targeting, assembly, degradation, or function of the ATP synthase subunits in PPT1-deficient cells. These aspects were analyzed next.

5.2.3 Mitochondrial function is unaltered in *Ppt1*$^{\Delta ex4}$ mouse brain (II)

Besides the subunit c accumulation, functional mitochondrial abnormalities have also been reported in NCL patients, including INCL patients, as well as animal models (discussed in Chapter 2.4.3). We wanted to examine the function of mitochondria in the brain of Ppt1-deficient mice in the light of our newly found interaction. For this purpose, we extracted the mitochondria from the cerebrum and cerebellum of two two-month-old wild type and *Ppt1*$^{\Delta ex4}$ mice, and examined by blue native electrophoresis whether the assembly of mitochondrial complexes was affected. The assembly of different respiratory complexes appeared normal after Coomassie staining (data not shown). To examine the amounts of nuclearly and mitochondrially encoded proteins, we compared the relative amounts of complex II (succinate – coenzyme Q reductase) and complex V (ATP synthase). Complex II is exclusively encoded by the nuclear DNA, while other respiratory complexes also contain mitochondrially encoded subunits (Zeviani and Di Donato 2004). No differences were found in their relative amounts compared to the wild type (II, Figure 3A). Furthermore, we tested the oxygen consumption of freshly isolated mitochondria from *Ppt1*$^{\Delta ex4}$ mouse brain, and detected no alterations compared to the wild type (II, Figure 3B). Even though mitocondria have been suggested to play a role in NCL neurodegeneration, our analyses on the mouse brain did not reveal any major failure in the assembly or function of ATP synthase or other respiratory complexes. It is possible that the alterations in mitochondrial function are subtle and may not cause abnormalities in complex assembly or function, at least when analyzed at the level of the whole brain tissue. As an example, defects in complex I in Parkinson's disease are demonstrated specifically in the substantia nigra of the human brain (Muqit et al. 2006, Schapira 2002). Therefore, in order to investigate the role of mitochondria in the neurodegeneration of INCL, temporal and spatial analyses of mitochondria in different brain areas, especially in the thalamus, should be conducted. At this point, we continued to examine other possibilities for the interaction between PPT1 and the F_1-complex in order to identify its significance.

5.2.4 Increased amount of ectopic F_1-ATP synthase in *Ppt1*$^{\Delta ex4}$ mouse neurons (II)

In addition to mitochondria, F_1-subunits have been found on the surface of different cell types. Studies reporting ectopic F_1-subunits include data from adipocytes, hepatocytes, and endothelial cells (Kim et al. 2004, Martinez et al. 2003, Moser et al. 2001). Additionally, different proteomic studies have reported F_1-subunits in lipid rafts extracted from the plasma membrane of various organs, including the brain (Bae et al. 2004, Kim et al. 2006a). However, no reports are available describing neuronal ectopic F_1-subunits. We utilized cell surface biotinylation and TIRF micrscopy to investigate the cell surface localization of F_1-subunits in mouse fibroblasts and neurons. F_1-subunits α and β were detected on the surface of both cell types, and for the first time on the surface of neurons (II, Figure 5). Western blot and immunofluorescence analyses showed that the overall amount of these proteins was similar in wild type and *Ppt1*$^{\Delta ex4}$ mouse cells. When the ectopic amounts of F_1-subunits were compared with TIRF microscopy, an increase in *Ppt1*$^{\Delta ex4}$ neurons was detected (II, Figure 6). However, no difference was detected between the mutant and control fibroblasts.

To examine the functional link between the increase in ectopic F_1-subunits and the depalmitoylating activity of PPT1, we treated the wild type cells with 2-bromopalmitate (2BP) to inhibit palmitoylation. Our first hypothesis was that if the increase was due to decreased depalmitoylation of proteins by PPT1, then the inhibition of palmitoylation would further decrease the amount of ectopic F_1. However, no decrease in the wild type cells could be seen after overnight treatment with 10-100 μM 2BP (II, Figure 6). Palmitoylation can be either a constitutive or regulated modification, and the 2BP-procedure has been utilized in examining the regulated palmitoylation of cytosolic proteins PSD-95 (post-synaptic density protein 95) and Fyn (Src family kinase) (El-Husseini Ael et al. 2002, Webb et al. 2000). F_1-subunits are normally membrane-associated through an association with the membrane-embedded F_0-complex. It is not known whether the whole F_1-F_o-complex is present at the neuronal plasma membrane, or if only the F_1-complex or even the individual F_1-subunits possess their own ectopic functions. Also unknown is how they might be in contact with the membrane. In the light of the reported association of the F_1-subunits with lipid rafts, one explanation for the increased cell surface localization could be the reported defects in receptor-mediated endocytosis in Ppt1-deficient cells (Ahtiainen et al. 2006). Additionally, defects in saposin trafficking and cholesterol metabolism could cause general alterations in the membrane composition and influence, for example, the number of lipid rafts in Ppt1-deficient cells (Ahtiainen et al. 2007).

The function of the ectopic F_1-subunits has been studied in hepatocytes, adipocytes, and endothelial cells. Depending on the cell type, they can either hydrolyze or synthesize ATP. In adipocytes, the amount of F_1-subunits was increased during adipogenesis and they participated in extracellular ATP synthesis (Kim et al. 2004). Ectopic F_1-subunits also synthesized ATP in endotelial cells (Moser et al. 2001), while in hepatocytes they hydrolysed ATP and the resulting ADP was shown to activate the $P2Y_{13}$ receptor and trigger HDL endocytosis (Jacquet et al. 2005, Martinez et al. 2003). Many molecules have been shown to act as ligands for F_1-subunits (Champagne et al. 2006). For example, the β-subunit has been identified as a receptor for apoA-I, β-casomorphin, enterostatin, angiostatin, and circulating factor 6 (CF6). Endothelial monocyte activating polypeptide II (EMAP II) was identified as a ligand for the α-subunit. Ectopic F_1-subunits are thus implicated in various processes, such as lipid metabolism, angiogenesis, blood pressure regulation, and tumor recognition by innate immune cells. Their function in neurons is not known. However, previous studies from our group addressed the dysregulation of cholesterol metabolism in *Ppt1*$^{\Delta ex4}$ mouse neurons (Ahtiainen et al. 2007).

Neuroinflammation is the hallmark of INCL, suggesting a disruption of astrocyte-neuron crosstalk. Astrocyte-neuron interactions are also essential for neuronal cholesterol trafficking and therefore, in order to analyze the function of ectopic F_1-subunits in neurons and astrocytes, we decided to measure the uptake of lipid-poor apoA-I. This protocol was chosen as the β-subunit had been identified as the receptor for apoA-I in hepatocytes, and apoA-I is one of the apolipoproteins present in cerebrospinal fluid (CSF). Ppt1-deficient cells were incubated with an excess of radiolabelled apoA-I, and the amount of internalized apoA-I was compared to wild type cells. The overall uptake was low, but a significant increase in the amount of internalized apoA-I was detected in Ppt1-deficient neurons and glial cells (140% and 118% of wild type, respectively) (II, Figure 7). This result suggested a possible connection between the elevated levels of β-subunit on the cell surface and the increased uptake of apoA-I. Further studies are needed to elucidate the precise function of the ectopic F_1-subunits in neurons in order to clarify their role in ATP/ADP metabolism. P2Y and P2X receptors (or P2 purinoceptors), activated by ADP, ATP, and other nucleotides, have recently been linked to many different functions in the CNS. One of them is CNS development (Zimmermann 2006). The proliferative capacity of Ppt1-deficient progenitor cells has been reported to be enhanced (Ahtiainen et al. 2007). Because purinergic signalling has been reported to sustain and enhance the proliferation of neural progenitor cells (Lin et al. 2007), there may be a link between the two. Purinoceptors seem especially important to glial cells in intercellular communication, and misregulation of these receptors has been implicated in diverse pathological conditions, including microglial activation after neuronal injury and disease-linked neurodegeneration (Franke and Illes 2006,

Inoue et al. 2007). Altered nucleotide signaling might thus play a role in INCL pathogenesis as well.

5.2.5 Evidence of dysfunction in cellular and systemic lipid metabolism in *Ppt1*$^{\Delta ex4}$ mice

Next, it would have been of significant interest to us to compare the lipoprotein levels in the CSF of *Ppt1*$^{\Delta ex4}$ and wild type mice. Alterations in CSF lipoprotein levels have been reported in patients with neurodegenerative diseases (Michikawa 2004, Vuletic et al. 2005). However, sufficient CSF samples are very difficult to obtain from mice because of their small size. Therefore, we decided to compare various serum lipid parameters between the wild type and Ppt1-deficient mice, since in addition of being present in the CSF, apoA-I is the major apolipoprotein in plasma HDL. The amounts of cholesterol, triglycerides, apoA-I, and apoE, as well as the activity of plasma phospholipid transfer protein (PLTP), were investigated in 1-month-old mice. Interestingly, cholesterol, PLTP, and apoA-I levels were significantly reduced in Ppt1-deficient mice (II, Figure 8). PLTP transfers phospholipid-rich surface remnants from hydrolyzed triglyceride-rich lipoprotein particles to HDL, modulating its size and composition (Qin et al. 2000). No differences in the apoE serum levels were detected. However, a significant decrease in the particle size of apoE-rich HDL lipoproteins was detected. Disturbances in the function of apoE have been studied in the apoE-deficient mouse model. These mice develop atherosclerosis and signs of neuronal deficits, mirroring the results of apoE dysfunction in humans (Moghadasian et al. 2001, Veinbergs et al. 1998).

Reduced adiposity, cholesterol levels, and triglycerides were recently reported in the INCL mouse model as well as in several other mouse models of lysosomal storage diseases (Woloszynek et al. 2007). Lysosomal storage *per se* was hypothesized to cause energy depletion, because cells lack the nutritients recycled by lysosomes, and, in addition, they have to "maintain" the storage. The reduced cholesterol and lipoprotein levels we detected may well reflect the alterations in the energy balance. The only human study reporting abnormal lipoprotein profiles in NCLs was done with JNCL patients and dates back to the 1980's (Bennett et al. 1986). Bennett and co-workers found decreased levels of the VLDL fraction in patients' sera. Since VLDL is the major lipoprotein carrier for triglycerides, their results could also reflect reduced adiposity and energy depletion due to lysosomal storage. The reduction in particle size of the apoE-containing HDL lipoproteins has not been linked to energy balance, but may reflect a disturbance in the maturation and subclass distribution of HDL and has been connected to the apoE4 genotype in human studies (Dart and Cooper 1999, Topic et al. 2008).

5.3 Interaction of PPT1 with CLN5 (III)

5.3.1 Interactions of CLN5 with five other NCL proteins

NCL diseases resemble each other both clinically and histopathologically. However, eight different genes have already been identified behind these diseases, and it has been estimated that at least two more genes wait to be identified (Siintola et al. 2006). Therefore, it is tempting to hypothesize that NCL proteins could be functionally connected and participate in one common pathway at different levels. It was previously known from the GST-pull down experiments that CLN5 interacts with two other NCL proteins, CLN2/TPP1 and CLN3 (Vesa et al. 2002). One study also described that NCL proteins could complement each other and correct growth and apoptotic defects in mouse fibroblasts (Persaud-Sawin et al. 2007). To elucidate the possible functional connections between different NCL proteins, we utilized the GST-CLN5 pull down assay to fish endogenous NCL proteins from mouse brain extract and HeLa lysate. GST-vector and other lysosomal/ER proteins were used as controls. We were able to replicate the interactions between CLN5 and CLN2/TPP1 and CLN3 (III, Figure 1). In addition, we detected three novel interactions between CLN5 and other NCL proteins when PPT1, CLN6, and CLN8 precipitated with CLN5. We could not detect the endogenous CLN8 to bind to CLN5, but when CLN8 possessing a HA-tag was overexpressed in COS-1 cells, an interaction was observed. LAMP-1 and cathepsin D were negative for CLN5 interaction. To conclude, CLN5, a soluble lysosomal protein, showed interactions with two other soluble lysosomal enzymes, PPT1 and TPP1, one endo/lysosomal transmembrane protein, CLN3, as well as with two ER transmembrane proteins, CLN6 and CLN8. The detected interactions between the ER-resident and lysosomal proteins could be explained by the possible actions of the ER-proteins on CLN5, as the post-translational modifications or folding of CLN5 could be modified by CLN6/CLN8. Another possibility is that CLN5 already has a function in the ER, and CLN6 and CLN8, and possibly other interaction partners as well, are the objects of this function. In either case, the hypothesis of a common pathway affected in NCLs is supported by the multiple interactions of CLN5 with other NCL proteins.

5.3.2 Effects of the PPT1 – CLN5 interaction on their transport

The major question following the detected interactions was whether they had any consequences at the cellular level. This issue was first approached by examining the intracellular localizations of the interacting proteins in the absence of the Cln5 protein. Interaction partners were transiently expressed in Cln5-deficient mouse

fibroblasts and detected by immunofluorescence staining. No difference was detected in the intracellular localization of the interacting NCL proteins (data not shown). Next, we analyzed their localization when co-expressed with CLN5 in HeLa cells. The intracellular localizations of the analyzed NCL proteins again remained unchanged, although CLN3 was often detected in the ER in addition to lysosomes. CLN5 was also partly retained in the ER with CLN3, possibly indicating a defect in their folding due to strong expression and the hydrophobicity of the transmembrane protein CLN3 (data not shown).

We proceeded to analyze whether the disturbances in the intracellular trafficking of CLN5 would affect the trafficking of the interaction partners. To this end, we utilized transient overexpression of a C-terminally flag-tagged CLN5 construct, which is unable to leave the ER. Co-expressions were performed in HeLa cells. Interestingly, the wild type PPT1 was retained in the ER when co-expressed with CLN5-flag (III, Figure 2). To control the experiment, PPT1 was also co-expressed with a trafficking-deficient flag-CLN3 (data not shown) and CLN6, an ER-protein. When co-expressed with these proteins, PPT1 was able to leave the ER and was transported to lysosomes. The localization of the other interaction partners was normal with CLN5-flag, although again a fraction of CLN3 was retained in the ER (III, Figures 2 and 3). The total arrest in PPT1 transport was a strong indication of the importance of the interaction between CLN5 and PPT1, and we focused our further analyses on dissecting this observation.

The ER-resident $PPT1_{Fin}$ mutant was co-expressed with the wild type CLN5 to investigate whether the transport defect was bilateral, i.e. whether trafficking-deficient $PPT1_{Fin}$ could retain the wild type CLN5 in the ER. However, during the co-expression of wild type CLN5 and $PPT1_{Fin}$, both polypeptides showed characteristic distribution in the majority of the experiments – wild type CLN5 was able to traffic to the lysosomes while $PPT1_{Fin}$ retained in the ER (III, Figure 4). Another CLN5 polypeptide deficient in trafficking is the $CLN5_{FinMajor}$ protein, which carries the most common Finnish mutation (c.1175delAT, Y392X) (Savukoski et al. 1998). This mutant protein has been shown to localize in the ER and Golgi region but not in lysosomes (Isosomppi et al. 2002). The mutation results in an early stop codon 16 amino acids before the C-terminus and consequent truncation of the polypeptide chain. When the wild type PPT1 was co-expressed with the mutated CLN5, the result was intriguing – PPT1 was observed to cluster into perinuclear vesicles, opposite to its normal, wide-spread vesicular distribution (III, Figure 4). The perinuclear vesicles where PPT1 was clustering were further analyzed in HeLa cells and in human neuroblastoma SH-SY5Y cells. They were proven to be mostly of late endosomal/lysosomal origin, as LAMP-1 colocalized with PPT1 (III, Figure 5). Sometimes PPT1 retention was stronger and it partially co-localized with the Golgi

marker GM-130 as well (**Figure 9**). More importantly, wild type PPT1 was able to facilitate the transport of the $CLN5_{FinMajor}$ polypeptides from the ER and Golgi region to the lysosomes, as $CLN5_{FinMajor}$ was also partly colocalizing with the PPT1/LAMP-1 positive vesicles in addition to the localization in the ER and Golgi (**Figure 9**) (III, Figure 5). Furthermore, there seemed to be a correlation between the intensity of $CLN5_{FinMajor}$ expression and colocalization. When the $CLN5_{FinMajor}$ expression level was low, almost the entire signal appeared vesicular. When the expression was strong, most of the signal was seen in the ER. Without PPT1 co-expression, $CLN5_{FinMajor}$ was detected only in the ER and Golgi, as previously reported.

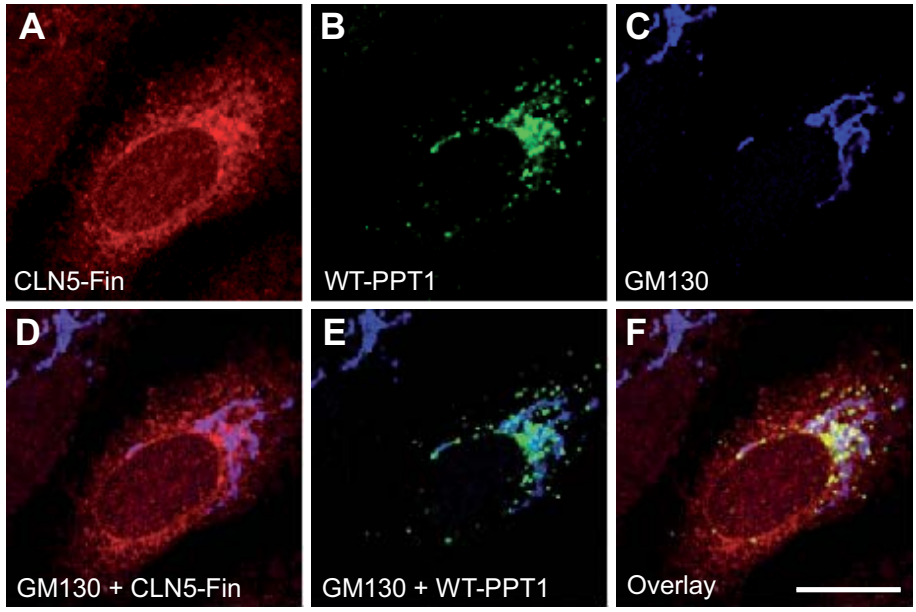

Figure 9. ***Co-expression of $CLN5_{FinMajor}$ (A) and wild type PPT1 (B) in HeLa cells labeled with the Golgi marker GM130 (C). GM130 co-localizes partially with $CLN5_{FinMajor}$ (D) and PPT1 (E). In the overlay image (F), the colocalization of CLN5FinMajor and PPT1 is shown in yellow, and the colocalization of all three proteins in white. Scale bar 10 µm.***

Consequently, our results implicated a role for PPT1 in CLN5 transport. The arrest of PPT1 transport in the ER when expressed with CLN5-flag possibly results from the strong interaction between the two proteins. However, its functionality cannot be assessed with these experiments. Regardless, as CLN5 is able to leave the ER without PPT1 most of the time, or the mutated PPT1 does not restrain its trafficking, it is the action of CLN5 on PPT1 that seems to be the important function in the ER. The relationship seems to be reversed in the Golgi region, where PPT1 facilitates CLN5. It is also possible that the clustering of PPT1 may result from dysfunctional CLN5. In any event, as both NCL proteins studied here are soluble and intravesicular, it is likely that the effect seen in their trafficking comes across via a third transmembrane protein functioning in the trans-Golgi region and participating in protein sorting events. The ability of PPT1 to enhance the lysosomal transport of the mutated CLN5 resembles the newly reported transport route for β-glucocerebrosidase. Interestingly, the transport of the mutant β-glucocerebrosidase is enhanced by the overexpression of LIMP-2 (Reczek et al. 2007). However, the transmembrane protein, or the mechanism of how the information about the function of PPT1 and/or CLN5 affects the cytosolic face of the transport vesicle, remains undefined. A very interesting aspect here is the role of sortilin, another receptor for lysosomal transport and a protein participating in the trafficking of saposins. The storage material in INCL (and minor amounts in $vLINCL_{Fin}$ as well), together with the reported delay in the transport of saposins A and D in Ppt1 deficient cells (Ahtiainen et al. 2006), offer a very good basis for studying the relationships between these three proteins in the future.

5.3.3 The interaction between CLN5 and the F_1-ATP synthase

Because the interaction between CLN5 and PPT1 was strong, it was of interest to investigate whether CLN5 would also interact with the previously reported binding partner of PPT1, the F_1-ATPase. Indeed, GST-CLN5 pull down analysis showed both F_1-subunits, α and β, to bind to CLN5 (III, Figure 6). To analyze if the two NCL proteins required each other in order to interact with the F_1-subunits, the pull downs were performed from mouse tissue extract derived from the Ppt1- and Cln5-deficient mice. The interactions were positive in these conditions as well, implying that the proteins interact with the F_1-ATPase independently. Disturbances in cellular and systemic lipid metabolism were observed in the Ppt1-deficient mice, and future studies will hopefully show whether the same is true in Cln5 deficiency. It will also be crucial to clarify the role of ectopic F_1-ATPase in neuronal function, as discussed above.

In summary, these results show that NCL proteins are connected at the molecular level, proposing a central role for CLN5 in the "common NCL-pathway" (**Figure**

10). Moreover, PPT1 was able to partially correct the transport of mutant CLN5. Finally, CLN5 was shown to interact with the F_1-ATPase, thus linking PPT1 and CLN5 more tightly together functionally.

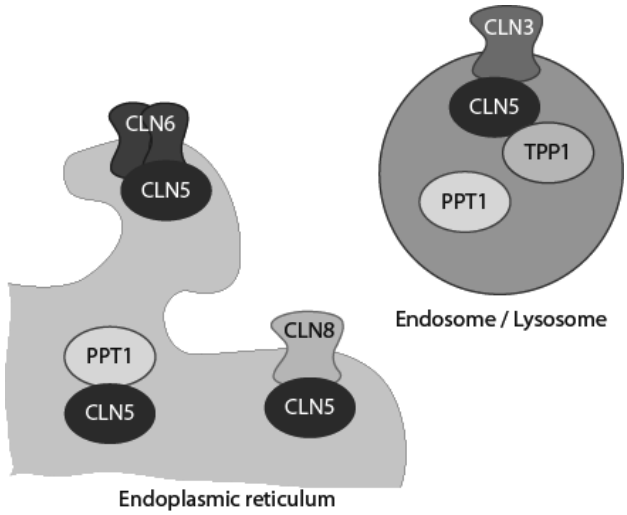

Figure 10. ***The interactions between CLN5 and other NCL proteins described here. The interaction between CLN5 and PPT1, CLN8, and CLN6 occurs in the ER. The location of interaction between CLN5 and TPP1 or CLN3 is unknown.***

6 CONCLUSIONS

The genes behind INCL and vLINCL$_{Fin}$ were characterized over a decade ago, and since then, a great amount of knowledge has been gained as far as understanding the molecular biology of these diseases. When this study was initiated, the function and three-dimensional structure of PPT1 had been described. It was known that PPT1 was developmentally regulated in the human and mouse central nervous system, and the storage material accumulating in PPT1-deficient cells had been identified. PPT1 had already been characterized extensively in non-neuronal cells, and studies examining PPT1 in neurons had been initiated. In addition, analyses on different disease mutations behind variable phenotypes had been initiated. However, the possible substrates of PPT1, or proteins functionally linked to PPT1, had not been identified *in vivo*. This work was initiated in order to characterize the neuronal properties and molecular interactions of PPT1, as well as to characterize the interactions of CLN5 with other NCL proteins.

The major findings of this study were the identification of the F_1-ATP synthase as an interaction partner of PPT1 and CLN5, as well as, on a more specific level, the neuron-specific alterations in the amounts of F_1-subunits in Ppt1-deficient cells. Naturally, the results raise a cascade of further questions that need to be answered before the functional relevance of this interaction can be explained. However, initial evidence for a disturbance in lipid metabolism was detected in Ppt1-deficient cells of the central nervous system, together with alterations in the pattern of plasma lipid components. These findings are supported by previous results from gene expression studies and functional experiments performed on mouse neurons, describing alterations in cholesterol biosynthesis in young Ppt1-deficient neurons (Ahtiainen et al. 2007). It will be of significant interest to analyze the parameters of lipid metabolism in Cln5-deficient neurons and plasma, and to determine whether similar aberrations are to be found in Cln5- and Ppt1-deficient mice. Further characterization of the defects linked to the disturbed lipid metabolism in NCL may also yield novel insights into the fundamental properties of neurons. It is likely that in the near future it will be possible to dissect whether the initial set of events in NCL pathogenesis are due to a dysfunction in neuronal development or whether this group of diseases is better characterized by neuronal degeneration, as originally assumed.

Another interesting finding of this work was the rather strong interaction between PPT1 and CLN5, which affects the intracellular transport of the proteins. Given the specific involvement of the CNS in the pathogenesis of NCL disorders when compared to other lysosomal storage disorders, the intracellular membrane trafficking in neurons in general may turn out to be a salient point in these diseases.

NCL proteins should thus be kept in mind when dissecting the mechanisms of neuronal trafficking. Gene expression studies from Ppt1- and Cln5-deficient mouse cortices support the importance of the detected interaction between PPT1 and CLN5, since similar biological pathways were affected in both disease models (von Schantz et al. 2008). In this light, it will be interesting to in more detail also characterize the interactions between CLN5 and other NCL proteins described here.

In this work, specific neuronal features of PPT1 were described. PPT1 was found to be differentially modified in neurons and fibroblasts, and to traffic differently from another lysosomal enzyme, AGA. These observations may provide clues for the altered intracellular localization of PPT1, although more work is needed to explain the eccentricities of neuronal trafficking *per se* before the underlying cellular events causing the death of neurons in PPT1 deficiency are clarified. The vital role of glial cells in the development and survival of neurons has only recently been recognized, and may prove to be crucial in NCLs as well as in other neurodegenerative diseases. This is supported by the link to lipid metabolism and altered particle size of apoE-containing HDL lipoproteins in the Ppt1-deficient mouse sera, as the same apolipoprotein is linked to Alzheimer's disease as well. It may turn out that the initial problem in NCL diseases is the cross-talk between neurons and glial cells rather than that between neurons.

In spite of both diseases being enriched in Finland, researchers in the US as well as in other parts of Europe have done much work to further our understanding of INCL, whereas Finns have up until recently been responsible for most of the $vLINCL_{Fin}$ research. European collaboration funded by the EU, established to combine the powers of many small NCL research groups in Europe, was ongoing concurrently with this thesis work. This collaboration has been beneficial especially in advancing our understanding of the initial events in NCL disorders, and it initiated the systematic analyses of different NCL diseases (Cooper et al. 2006). These analyses continue, as high-throughput projects comparing different NCL diseases are on-going in an effort to elucidate their similarities and differences in terms of gene expression, metabolomics, and lipidomics (Jalanko et al. 2006). Collaborations between different fields of specialties are needed in the future when aiming to decipher the disease mechanisms behind NCL diseases.

7 ACKNOWLEDGEMENTS

This thesis work was carried out at the Department of Molecular Medicine, National Public Health Institute, during the years 2002-2008. I wish to acknowledge the former and present directors of the Institute, Jussi Huttunen and Pekka Puska, and the Heads of the Department, Leena Palotie, Christian Ehnholm and Anu Jalanko, for providing excellent research facilities.

Financial support was provided by the Science Foundation of the University of Helsinki, Rinnekoti Foundation, Arvo and Lea Ylppö Foundation, Oskar Öflund's Foundation, the Finnish Medical Association, Sigrid Juselius Foundation, Academy of Finland and the European Commission, which are gratefully acknowledged.

Professors Thierry Levade and Pirjo Laakkonen are warmly thanked for the careful revision of my thesis, and for their constructive suggestions to improve the manuscript. I wish to thank Professors Elina Ikonen and Anu Wartiovaara for their participation in my thesis committee and for the advice and encouragement during the final steps of my work. Anu W. is further thanked for the fruitful collaboration and for sharing her expertise concerning mitochondria, as well as accepting the role of kustos at my thesis defence. I am grateful to Professor Mart Saarma for accepting the role of opponent in my thesis dissertation.

I am grateful to my supervisors, Adjunct Professors Anu Jalanko and Aija Kyttälä, for all the guidance and support during these years. Anu, I owe my deepest gratitude to you for trusting in me and my capabilities. The independence you have given me, has on one hand offered me the privilege to work flexibly and to keep up with med school, and on the other hand, has made me work harder than ever. I have always felt welcome to visit your office and discuss any matter regarding science or life in general, and I have also felt that my opinions were listened to. Aija, without your input to my project I would not be here today. Your determination has been invaluable in terms of getting it all together. I also wish to thank you for sharing your expertise in cell biology, and for the fun times.

I wish to express my gratitude to all the co-authors and collaborators. Matti Jauhiainen is heartily thanked for acting as my biochemistry mentor and for being a great collaborator during these years. Matti's positive attitude, his amazing (and infectious!) enthusiasm towards science, and our educational discussions have given me energy to continue my work and spared me from many moments of frustration. Tarja Salonen is thanked for guiding me at the early stages of this project and for introducing me into the world of PPT1. Jani Saarela is thanked for teaching me all the main experimental protocols concerning proteins, and for the continuous interest towards my project. Outi Kopra is thanked for sharing her expertise in neuroscience and immunohistology, and for her valuable comments on my manuscripts, including this thesis. Sanna Marjavaara is warmly thanked for all the effort and skilful work in the mitochondrial analyses, as well as for the entertaining discussions about life and science during those early hours in the lab. Kimmo Tanhuanpää is warmly acknowledged for his input in the TIRF-imaging analyses that made the second study so much better. Nisse Kalkkinen is thanked for sharing his broad knowledge in mass spectrometry with students like me. Laurent Martinez is greatly acknowledged for providing the purified ATP synthase protein samples for our experiments, and for his genuine interest towards our projects.

Claudia Heine is warmly thanked for her hard and persistent work with the CLN5 protein, which set the ground for the third publication.

I wish to thank Carina von Schantz-Fant and Kristiina Uusi-Rauva for sharing the journey of a PhD student in the NCL-group with me from the very beginning. Without your peer support, I would have been lost. Tintti, you have shared your positive attitude, loud and long phone calls, the Love Songs on Fridays, top organizational skills, the Finnish Swede social life, two publications, and your true friendship with me. It has been invaluable and made this journey so much more fun. Kristiina, I am indebted to you for your determination and skill in problem-solving concerning various experiments and other scientific issues. Your helpful attitude, our long conversations before heading home, and your contagious laugh have made these years much more enjoyable.

I also wish to thank all the past and present members of the AGU-NCL group: Kaisu, Laura, Ville, Juha I, Kaitsu, Jani, Liina, Minna, Salli, Mia S, Elina, Mari and Tessa are thanked for the great company. Kaisu and Laura are especially thanked for sharing the office, the conference trips and for being great examples of what a PhD student should become like. Mia is thanked for her positive company at work and parties, and for taking the CLN5 project in her skilful hands. Our technicians, Auli, Kaija, Tuula, Essi, Anne N., Paula and Marja, are warmly thanked for all the help in the lab. Auli, I am grateful to have had the opportunity to work with you. Our team work has been crucial for the progress of this project. Thanks for your flexibility, and your patient and analytical attitude towards the not-always-so-straight-forward protein experiments. Other members of the "big lab" and MLO in general are also acknowledged for their input and nice atmosphere. Thanks to Mira, Jonna, Heli, Heidi N, Heidi L, Heidi M, Elina, Niklas, Anna, Juha P, Annika, Nabil, Heli R, Mikko K, Tero Y, Jenni, Henna, Jonas, Nora, Maria, Taina, Tanja, Markus, Anne V., Lennu, Ritva, Katta, Outi, Heli K, Riikka H, Riikka V, Mikko M, Jari, Sari, Seikku, and all the people I have forgotten to mention.

Scientists in our department and elsewhere are thanked for their support in various matters. I would like to thank the senior scientists Ismo Ulmanen, Marjo Kestilä, Jaana Vesterinen, Janna Saarela, Vesa Olkkonen, Massimiliano Gentile and Teppo Varilo for advice and support. Iski was the person who converted me from organic chemistry to biochemistry for good by suggesting a gradu project in Anu's group – a change I haven't regretted once. I wish to thank Marjo for her firm, yet calm attitude which helped me cope with the tight schedule and the bureaucracy. Jaana is thanked for the collaboration and help with various NCL-related issues. Mia Lindfors is thanked for sharing the unforgettable experience of proteomics. Fellow NCL-students Mia, Eija, Mervi K, Eeva, Aleksi, Carlos and Sanna are thanked for their company. Sari M, Sari K, Tuija, Sanna, Sisko and Mika are acknowledged for their administrative help, and Jari, Juri, Teemu, Tero, Sampo and Juho for the IT-support. Tero, Juri and Sampo are also thanked for great company at numerous parties. Ville Järvi is thanked for the careful language revision of this thesis.

Our coffee group has varied its composition over the years, but it has always offered an amusing break from the work. I want to thank Kirsi, Mervi, Kati, Johannes, Olli, Marika, Jussi, P-P, Emma P, Emma N, Joni, Pekka, Minna, Liisa, Johanna, Will, Jenny, Jesper, Tony, Dennis, Iiris, Teppo, Päivi, Annu, Minttu, Pia, Juho and many more, for the cheerful moments. Will is additionally thanked for the title of this book, and Pekka for answering all my dumb questions about sequencing.

I wish to thank Cursus Intrepidus for all the fun years in med school, and for your open-minded attitude towards an oldie like me. I am happy there is still much more to come. My special thanks go to Anna, who has become a very close friend during these years. Thank you for your continuous support and amazing company. I also want to thank all my friends outside the lab or med school for giving me the perspective of what life really is. Mirka and Laura have been especially important to me. Thanks for all the help and for always being there when I needed it. I also want to send my apologies to Kerttu and Else for not having been a very good godmother, yet. I hope by the time you can read this, you'll wonder what I was talking about.

I would like to thank my family, grandparents and relatives for their encouragement. My future family-in-law, Tarja, Anssi, Minna and Miika, are thanked for their support and interest towards my work, and for all the good moments in Finland and abroad. Minna is especially thanked for the assured understanding. I wish to thank my parents Olavi and Eija for their unconditional love and support, and for always believing in me. My siblings Auri and Lauri are thanked for sharing and caring. Auri and my sister-in-law Hanna are further thanked for their friendship and all the invaluable moments in Somero.

Finally, I owe my deepest gratitude to Markus. Thank you for all the love and happiness you have brought to my life. Without you nothing would matter.

In Helsinki, May 8th, 2008

Annina Lyly

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