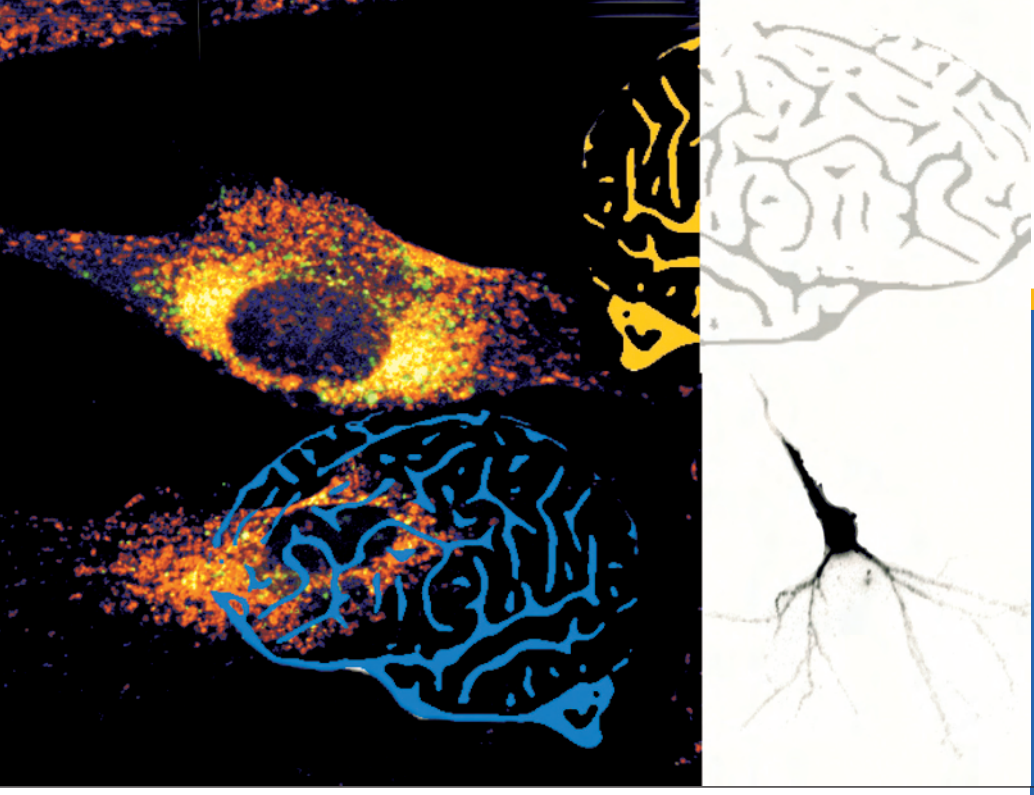

Laura Ahtiainen

Unravelling Molecular and Cellular Disease Mechanisms in Infantile Neuronal Ceroid Lipofuscinosis (INCL)

Publications of the National Public Health Institute A 5/2007

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and
Helsinki Graduate School in Biotechnology and Molecular Biology,
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Helsinki 2007

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UNRAVELLING MOLECULAR AND CELLULAR DISEASE MECHANISMS IN INFANTILE NEURONAL CEROID LIPOFUSCINOSIS (INCL)

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 Faculty of Biosciences of the University of Helsinki, for public examination in the Niilo Hallman lecture hall, Hospital for Children and Adolescents Helsinki University Central Hospital, on May 11th, 2007, at 12 noon.

Department of Molecular Medicine, National Public Health Institute, Helsinki, Finland

and

Helsinki Graduate School in Biotechnology and Molecular Biology,
Faculty of Biosciences, University of Helsinki, Finland

Helsinki 2007

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Publications of the National Public Health Institute
KTL A5 / 2007

Julkaisija-Utgivare-Publisher

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ISBN 978-951-740-696-3
ISSN 0359-3584
ISBN 978-951-740-697-0 (pdf)
ISSN 1458-6290 (pdf)

Kannen kuva - cover graphic:
Laura Ahtiainen

Edita Prima Oy
Helsinki 2007

“Equipped with his five senses, man explores the universe around him and calls the adventure Science.” ~Edwin Powell Hubble, The Nature of Science, 1954

Table of contents

List of original publications

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

I. **Ahtiainen L**, Van Diggelen OP, Jalanko A, Kopra O. Palmitoyl protein thioesterase 1 is targeted to the axons in neurons. J Comp Neurol. 455:368-77, 2003.

II. **Ahtiainen L**, Luiro K, Kauppi M, Tyynelä J, Kopra O, Jalanko A. Palmitoyl protein thioesterase 1 (PPT1) deficiency causes endocytic defects connected to abnormal saposin processing. Exp Cell Res. 312:1540-53, 2006.

III. **Ahtiainen L**, Kolikova J, Mutka AL, Luiro K, Gentile, M, Ikonen E, Khiroug L, Jalanko A, Kopra, O. Palmitoyl protein thioesterase 1 (Ppt1) deficient mouse neurons show alterations in cholesterol metabolism and calcium homeostasis prior to synaptic dysfunction. Submitted.

Abbreviations

3MA	3-methyladenine
aa	amino acid(s)
ANCL	adult neuronal ceroid lipofuscinosis (*CLN4*)
ATP	adenosine triphosphate
BA1	bafilomycin A1
bp	base pair
cDNA	complementary DNA
CHO cells	Chinese hamster ovary cells
CLN1	human *CLN1* gene
SCMAS	subunit c of mitochondrial ATP synthase
CNS	central nervous system
DIV	days *in vitro*
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EM	electron microscopy
IEM	immunoelectron microscopy
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate
GABA	γ-aminobutyric acid
GST	glutathione S-transferase
Hepes	*N*-2-hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid
HRP	horseradish peroxidase
Ig	immunoglobulin
INCL	infantile neuronal ceroid lipofus-cinosis (*CLN1*)
kb	kilobase(s)
kDa	kilodalton(s)
LINCL	late infantile neuronal ceroid lipofuscinosis
LSD	lysosomal storage disorder
MPR	mannose 6-phosphate receptor
mRNA	messenger RNA
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PI	phosphatidylinositol
PM	plasma membrane
PPT1	human palmitoyl protein thioesterase 1 protein
Ppt1	mouse palmitoyl protein thioesterase 1 protein
RNA	ribonucleic acid
RT	room temperature
SAP	sphingolipid activator protein
SDS	sodium dodecyl sulphate
SFV	Semliki Forest virus
SNAP	soluble NSF attachment protein
SNARE	SNAP receptor
SV	synaptic vesicle
TGN	*trans*-Golgi network
TRITC	tetramethylrhodamine isothiocyanate
vLINCL	variant form of late infantile neuronal ceroid lipofuscinosis (*CLN2*)
vLINCLFin	Finnish variant form of late infantile neuronal ceroid lipofuscinosis (*CLN5*)
wt	wild type

Laura Ahtiainen, Unravelling molecular and cellular disease mechanisms in Infantile Neuronal Ceroid Lipofuscinosis (INCL)
Publications of the National Public Health Institute, A5/2007, 109 Pages
ISBN 978-951-740-696-3; 978-951-740-697-0 (pdf-version)
ISSN 0359-3584; 1458-6290 (pdf-version) http://www.ktl.fi/portal/4043

Abstract

Studying neurodegeneration provides an opportunity to gain insights into normal cell physiology, and not just pathophysiology. In this thesis work the focus is on Infantile Neuronal Ceroid Lipofuscinosis (INCL). It is a recessively inherited lysosomal storage disorder. The disease belongs to the neuronal ceroid lipofuscinoses (NCLs), a group of common progressive neurodegenerative diseases of the childhood. Characteristic accumulation of autofluorescent storage material is seen in most tissues but only neurons of the central nervous system are damaged and eventually lost during the course of the disease leaving most other cell types unaffected. The disease is caused by mutations in the *CLN1* gene, but the physiological function of the corresponding protein the palmitoyl protein thioesterase (PPT1) has remained elusive.

The aim of this thesis work was to shed light on the molecular and cell biological mechanisms behind INCL. This study pinpointed the localization of PPT1 in axonal presynapses of neurons. It also established the role of PPT1 in early neuronal maturation as well as importance in mature neuronal synapses. This study revealed an endocytic defect in INCL patient cells manifesting itself as delayed trafficking of receptor and non-receptor mediated endocytic markers. Furthermore, this study was the first to connect the INCL storage proteins the sphingolipid activator proteins (SAPs) A and D to pathological events on the cellular level. Abnormal endocytic processing and intracellular re-localization was demonstrated in patient cells and disease model knock-out mouse neurons. To identify early affected cellular and metabolic pathways in INCL, knock-out mouse neurons were studied by global transcript profiling and functional analysis. The gene expression analysis revealed changes in neuronal maturation and cell communication strongly associated with the regulated secretory system. Furthermore, cholesterol metabolic pathways were found to be affected. Functional studies with the knock-out mouse model revealed abnormalities in neuronal maturation as well as key neuronal functions including abnormalities in intracellular calcium homeostasis and cholesterol metabolism.

Together the findings, introduced in this thesis work, support the essential role of PPT1 in developing neurons as well as synaptic sites of mature neurons. Results of this thesis also elucidate early events in INCL pathogenesis revealing defective pathways ultimately leading to the neurodegenerative process. These results contribute to the understanding of the vital physiological function of PPT1 and broader knowledge of common cellular mechanisms behind neurodegeneration. These results add to the knowledge of these severe diseases offering basis for new approaches in treatment strategies.

Keywords: neurodegeneration, lysosomal storage disease, neuronal ceroid lipofuscinosis, endocytosis, intracellular membrane trafficking, neuronal maturation, cholesterol metabolism, calcium homeostasis

Laura Ahtiainen, INCL-taudin (infantiili neuronaali seroidilipofuskinoosi) molekyyli- ja solutason tautimekanismit
Kansanterveyslaitoksen julkaisuja, A5/2007, 109 sivua
ISBN 978-951-740-696-3; 978-951-740-697-0 (pdf-versio)
ISSN 0359-3584; 1458-6290 (pdf-versio) http://www.ktl.fi/portal/4043

Tiivistelmä

Tutkimalla sairauden aiheuttamaa hermosolujen rappeutumista saadaan tietoa solujen toimintahäiriöiden lisäksi normaalista hermosolun toiminnasta. Tämä väitöskirjatyö käsittelee INCL-tautia (infantiili neuronaali seroidilipofuskinoosi). INCL-tauti on peittyvästi periytyvä lysosomaalinen kertymätauti. Se kuuluu neuronaalisten seroidilipofuskinoosien (NCL) tautiryhmään. NCL-taudit ovat melko yleisiä lapsuusiän eteneviä keskushermoston sairauksia, joille on tyypillistä autofluoresoivan materiaalin kertyminen useimpiin kudoksiin ja keskushermoston hermosolujen rappeuma. INCL-tauti aiheutuu virheistä *CLN1*-geenissä, mutta vastaavan palmityyli-proteiinitioesteraasi (PPT1) valkuaisaineen toiminta ja taudin syntymekanismit ovat huonosti tunnettuja.

Tämän väitöskirjatyön tavoitteena oli selvittää INCL:n taudinkuvan molekyyli- ja solutason mekanismeja. Tässä työssä kuvattiin PPT1-valkuaisaineen tarkka paikantuminen hermosolun viejähaarakkeen hermopäätteissä. Lisäksi selvitettiin PPT1:n merkitystä hermosolujen kypsymisessä ja kypsien hermosolujen toiminnassa. Kokeet INCL-potilaiden soluilla osoittivat häiriön solunsisäisessä kalvoliikenteessä, joka ilmentyi tiettyjen endosytoottisten merkkiaineiden hidastuneena kuljetuksena. Tässä työssä yhdistettiin ensimmäistä kertaa INCL-taudin kertymämateriaalin proteiinit, saposiinit A ja D, solutason patologisiin muutoksiin: Saposiinien solunsisäinen käsittelyn ja paikantumisen muutokset osoitettiin potilaiden soluissa ja muuntogeenisen hiirimallin hermosoluissa. Muuntogeenisen hiirimallin hermosoluja tutkittiin myös geenisiru-menetelmällä ja solun toimintaa kartoittavin menetelmin, aikaisten solutason aineenvaihduntareittien muutosten löytämiseksi. Nämä tutkimukset osoittivat muutoksia hermosolujen keskeisissä toiminnoissa: kolesteroliaineenvaihdunnassa, kalsiumin tasapainon säätelyssä, hermosolujen kypsymisessä ja solujenvälisessä viestinnässä.

Tässä väitöskirjassa esitetyt tutkimustulokset osoittavat PPT1-valkuaisaineen keskeisen merkityksen kehittyvissä ja kypsissä keskushermoston hermosoluissa. Tulokset selventävät aikaisia solutason tapahtumia INCL-taudin synnyssä, osoittamalla toimintahäiriöitä, jotka johtavat lopulta hermosolujen rappeutumiseen ja kuolemaan. Nämä tutkimustulokset auttavat suoraan PPT1:n normaalin solutason toiminnan ja myös laajemmin hermosolujen rappeutumisen aiheuttavien tekijöiden ymmärtämisessä. Lisäksi ne edistävät NCL-tautien toimintahäiriöiden ymmärrystä ja luovat uusia lähtökohtia hoitomenetelmien kehittämiseen

Avainsanat: neurodegeneraatio, lysosomaaliset kertymätaudit, neuronaalinen seroidi-lipofuskinoosi, endosytoosi, solunsisäinen kalvoliikenne, hermosolujen kypsyminen, hermosolujen kolesteroliaineenvaihdunta, kalsiumin tasapainon säätely

Review of the literature

1. Intracellular trafficking

1.1 Steps of cellular membrane trafficking

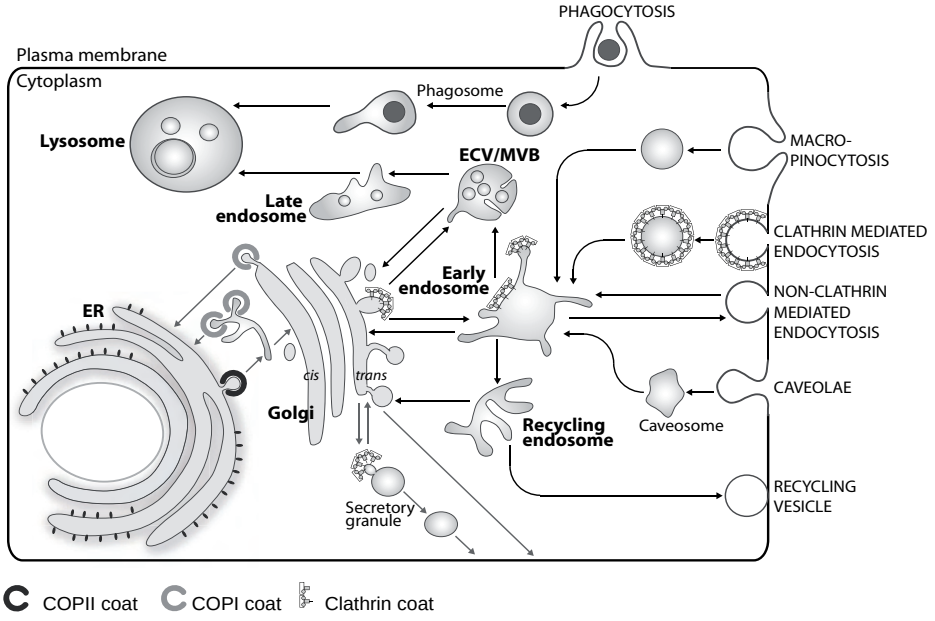
The eukaryotic cell is divided into compartments separated by membranes. The cell is separated from the external milieu by the plasma membrane and different cellular functions are carried out in membrane bound organelles. Communication between the cell and its surroundings as well as between different cellular organelles occurs via vesiculo-tubular carriers. The carrier vesicles have different lipid and protein compositions, depending on their specific role in the transport process. Membrane trafficking itself is a multi-step process starting with initiation of specific vesicle coat assembly and cargo selection, in which membrane embedded with proteins as well as soluble content proteins are specifically excluded or included in the vesicle. This is followed by budding of the vesicle from the donor compartment and targeting to the acceptor compartment. The vesicle carrier moves to the acceptor membrane along cytoskeletal tracks and upon contact with the acceptor membrane the vesicle fuses and unloads the cargo. Components involved with the vesicle formation and targeting are then released or recycled back to the donor compartment for a new round of employment (Bonifacino, and Glick, 2004; Mellman, and Warren, 2000; Olkkonen, and Ikonen, 2000; Olkkonen, and Ikonen, 2006).

1.2 Endosomes to lysosomes - Pathways of endocytic transport

The intracellular membrane traffic follows two main routes: the biosynthetic/secretory pathway and the endocytic pathway. These pathways are interconnected at several levels in the Golgi apparatus and the endocytic compartments (Bonifacino and Glick, 2004; Maxfield and McGraw, 2004; Soldati and Schliwa, 2006; van der Goot and Gruenberg, 2006). In the biosynthetic/secretory pathway newly synthesized proteins, carbohydrates and lipids are transported from the endoplasmic reticulum (ER) through the Golgi apparatus to the trans-Golgi network (TGN). The folding and post-translational modifications of newly synthesized proteins also occur during these transport steps. Biosynthetic/secretory pathway cargo is then directed to the cell surface or to the endosomes/lysosomes. The endocytic pathway comprises of uptake of

proteins, lipids, extracellular ligands, soluble molecules and pathogens discussed in detail below. The major pathways of intracellular membrane trafficking are represented in Figure 1.

Figure 1. Intracellular Membrane Transport Pathways.



Schematic representation of major intracellular membrane trafficking pathways. Transport steps are indicated by arrows.

In the biosynthetic pathway anterograde traffic takes place from the ER to the Golgi complex in COPII coated vesicles. Retrograde traffic occurs in transport intermediates coated with COPI complexes.

In the endocytic pathway the different cargoes are internalized through several pathways: in macropinocytosis, the clathrin-dependent and non-clathrin dependent pathways, internalized vesicles are transported to the early sorting vesicle the early endosome. In the caveolar pathway, marked by the caveolin coat (not shown) the cargo first travels to the caveosome compartment and then to the early endosome. Housekeeping receptors, for example the transferrin receptor binding transferrin and the low density lipoprotein (LDL) receptor binding LDL, are recycled to the plasma membrane directly or through recycling endosomes. Other cargo, in particular ubiquitinated signalling receptors that are being down regulated, are collected within forming endosomal carrier vesicles/multivesicular bodies (ECV/MVB). ECV/MVBs are then transported towards late endosomes with which they fuse. The cargoes that are destined for degradation and also soluble lysosomal and lysosomal membrane structural proteins continue to lysosomes, whereas others are recycled either to the plasma membrane (directly or through recycling endosomes) or to the Golgi apparatus the trans-Golgi network (TGN)(for example the cation-independent mannose-6-phosphate receptor (CI-MPR) binding lysosomal enzymes). The TGN destined cargoes, together with newly synthesized proteins, can exit the TGN and traffic either to the plasma membrane or to late endosomes or early endosomes.

Relatively large solid particles are taken up by phagocytosis into phagosomes, by an actin dependent process. Phagosomes progressively mature into phagolysosomes and then lysosomes.

Adapted from: van der Goot and Gruenberg, 2006; Soldati and Schliwa, 2006; Bonifacino and Glick, 2004; Maxfield and McGraw, 2004

1.3 Pathways of Endocytosis, internalization

Endocytosis is a cellular process for internalization of cargo from the exterior of the cell and transport to specific destinations inside the cell. Small essential molecules including ions, carbohydrates and amino acids can traverse the plasma membrane (PM) through specific channels, ion exchangers and adenosine triphosphate (ATP) powered pumps. However, larger macromolecules utilize a different mechanism, endocytosis, in which the plasma membrane invaginates into the interior of the cell forming transport vesicles. Endocytic uptake is divided into to two forms according to the endocytosed cargo: Phagocytosis, or cell eating, is initiated by the plasma membrane climbing up to encompass large cargo followed by intake. In pinocytosis/fluid phase endocytosis, or so called cell drinking, the membrane collapses inward. This process is actin dependent and involves the Rho family GTPases. (Conner and Schmid, 2003b; Perrais and Merrifield, 2005). The current view is that the plasma membrane contains distinct lipid and protein domains and these so-called hotspots favour specific types of internalization (De Matteis and Godi, 2004; Glebov, et al. 2006; Parton, et al. 2006).

1.3.1 Phagocytosis

Phagocytosis is the uptake of relatively large particles, 300 nm to several micrometers in diameter, such as large debris (e.g. apoptotic cells, deposits of fat) by an actin-dependent process. This pathway is also utilized by pathogens such as yeast and bacteria to gain access into the cell. The process of phagocytosis occurs most robustly in specialized cells such as macrophages, but most cells are capable of low levels of phagocytosis (Eskelinen, 2005). Receptors on the surface of phagocytic cells bind particles either directly or indirectly via antigen-bound antibodies. These receptors are capable of activating their own uptake and receptor binding triggers a signaling cascade involving activation of small GTPases, which results in actin assembly and formation of cell surface extensions. These actin- rich cellular extensions fuse up around the antigen and engulf it entirely forming a phagosome. The phagosome subsequently matures and fuses with lysosomes. (Conner and Schmid, 2003b; Eskelinen, 2005; Stuart and Ezekowitz, 2005)

1.3.2 The pinocytic pathways of internalization

Pinocytosis is divided in four different subtypes: macropinocytosis, clathrin-mediated endocytosis, clathrin-independent endocytosis and caveolae-mediated endocytosis. Macropinocytosis is the actin-dependent formation of large vesicles (from <0,2µm to over 1µm), which enable internalization of large quantities of fluid-phase solute from the extracellular milieu. In most cell types, an exogenous stimulus is required to induce macropinocytosis, however dendritic cells constitutively macropinocytose large quantities of exogenous solute as part of their function in the immune response (Norbury, 2006).

1.3.3 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis is a constitutive process in all mammalian cells. It is initiated at clathrin-coated pits that arise from pre-determined PM hotspots. The clathrin-coated pits are dynamic structures, undergoing exchange of coat protein components as they form allowing rapid production of multiple vesicles (Bonifacino and Lippincott-Schwartz, 2003; Edeling, et al. 2006; Kaksonen, et al. 2006; Perrais and Merrifield, 2005). The process of clathrin-coated vesicle formation can be divided into three steps 1) assembly of clathrin into a polygonal lattice and formation of coated pits, 2) invagination of coated pits, and 3) pinching-off of the coated vesicles. This process involves interactions of adaptor proteins with the plasma membrane and clathrin as well as several accessory proteins and phosphoinositides. Individual assembly units of clathrin, called triskelions, form lattices that assemble from a soluble pool of clathrin and associated adaptors to form a polygonal basket structure. The most abundant adaptor protein is the heterotetrameric protein AP-2 (adaptor or assembly protein-2)(Conner and Schmid, 2003a). Several accessory proteins are also implicated in the formation of clathrin-coated vesicles and recruiting AP-2 to the PM, such as AP180/CALM, Epsin, EPS15, dynamin and endophilin. In neuronal cells neurotransmitter secretion synaptotagmins have been proposed to act as general docking sites for coated pit formation (Diril, et al. 2006; Zhang, et al. 1994). The membrane factors that define the assembly sites include at least phosphoinositides. They are dynamically generated and broken down on the inner leaflet of the PM, and all adaptor proteins capable of promoting clathrin assembly have the ability to interact with

these lipids. PtdIns(4,5)P2 and PtdIns(3,4,5)P3 have been shown to be central for constitutive as well as induced endocytosis (Martin, 2001). Cholesterol is also assumed to contribute to invagination of coated pits since cholesterol depletion affects the ability of endocytic proteins, such as AP-2 and dynamin, to participate in lattice rearrangements to induce membrane invaginations (Subtil, et al. 1999). The multidomain GTPase dynamin has been implicated in multiple steps during clathrin mediated vesicle formation and it is central in mediating fission of the coated pit and release of the clathrin coated vesicle (Hill, et al. 2001). Cargo uptake to clathrin coated vesicles is regulated by several factors. Surface proteins can enter coated pits initially by chance and then be captured specifically by coated-pit components. Entry can also be achieved by binding to targeting proteins mediating selective transport to the coated pits. Receptor-mediated endocytosis can be either constitutive or stimulus induced. The sorting activity of the constitutive pathway is based on the recognition of a peptide sequence in the receptor termini. Examples of receptors utilizing the constitutive pathway include the transferrin receptor (TfR) and the low density lipoprotein receptor (LDLR). The signal-induced pathway sorting is mediated by adaptors and covalent protein modifications that facilitate receptor internalization. A well defined example of a receptor exploiting the signal induced pathway is a receptor tyrosine kinase (RTK) the epidermal growth factor (EGF) receptor. RTKs possess an intrinsic protein tyrosine kinase activity and upon ligand binding two monomeric receptors dimerize and conformation change is proposed to lead to exposure of endocytic motifs for endocytic adaptor binding (Burke, et al. 2001; Lemmon, et al. 1997).

1.3.4 Caveolin-mediated endocytosis

The caveolae are plasma membrane invaginations 50-80nm in diameter, coated by the caveolin-1, -2 and -3 proteins and rich in the ‘lipid raft’ components cholesterol and sphingolipids. It has been shown that caveolae- and lipid raft-mediated endocytosis plays roles in cell adhesion and anchorage-dependent cell growth; however underlying mechanisms are poorly understood. Cargo entering the cells via this pathway includes extracellular ligands (such as folic acid, albumin and interleukin-2), membrane components), glycosphingolipids and GPI (glycosylphosphatidylinositol)-anchored proteins and bacterial toxins. Also several viruses (Simian Virus 40, Polyoma Virus and Echovirus 1) employ this pathway to gain entry into the cells (Pelkmans, 2005).

Caveolae/raft-mediated endocytosis is thought to be initiated by the clustering of lipid rafts and the formation and/or sequestration into caveolae. This leads to increase of local cholesterol concentration inducing membrane curvature aided by concomitant clustering of proteins (presumably caveolin-1) associated with the cytosolic leaflet of lipid rafts. (Bacia, et al. 2005; Rothberg, et al. 1992). Also some evidence on virus entry studies suggest the existence of pre-assembled caveolar vesicles, termed caveosomes, fusing with the PM and mediating virus particle binding and entry (Pelkmans, 2005; Pelkmans and Zerial, 2005). In addition, caveolin dependent internalization also involves proteins regulated by phosphorylation of tyrosines by respective kinases leading to local actin polymerization and dynamin recruitment (Pelkmans and Zerial, 2005). The caveolae pinch off the PM and once inside the cell transfer their cargo into caveosomes that receive cargo exclusively from the caveolae mediated pathway. Inside the cell caveolar vesicles also dynamically dock on and fuse with the early endosomes, however in this kiss-and-run type interaction caveolar vesicles maintain their caveolin coat (Pelkmans, et al. 2004).

1.3.5 Clathrin- and caveolin independent internalization

Also endocytic pathways that do not require clathrin- or caveolin coats have been described. These pathways bypass the conventional endocytic organelles and converge with the caveolar pathway by utilizing the caveosomes as intermediates. It is not clear if these routes are independent cellular pathways or in fact variations of a common route. They are dependent on large amounts of the so called raft lipids. The rafts are highly ordered membrane microdomains within the bilayer. These ordered microdomains usually rich in glycosphingolipids and cholesterol are thought to appear independent of proteins and resulting from partitioning coefficients of particular lipid species (Simons and Toomre, 2000). Rafts by themselves cannot however be regarded as specific markers of these clathrin- and caveolin independent pathways since multiple endocytic mechanisms are used to internalize lipid raft components or molecules that preferentially partition into rafts (Pelkmans, 2005).

1.4 Transport along the endocytic route

At internalization to the cell the cargo enters the endosomal system. It is a network of organelles and vesicular intermediates that differ in biochemical composition and

localization within the cell. The carriers in the endocytic pathway, from endosomes to lysosomes, can be distinguished by several aspects including: a) morphology b) specific membrane, protein and lipid composition c) intralumenal pH d) the temporal dimension of a specific cargo reaching the compartment. A plethora of proteins are required for correct transport along the endocytic pathway but also membrane lipid microdomains, patches of membrane where certain lipids and proteins are enriched, formed locally on the compartments coordinate the transport process. The phosphoinositides (PIPs) are essential components in this process. They are distributed along the endocytic pathway in a spatially and temporally specific manner by specific phosphatidylinositol kinases and phosphatases. (Di Paolo and De Camilli, 2006; Lindmo and Stenmark, 2006; Perrais and Merrifield, 2005). Transport steps following the actin-dependent internalization process are primarily microtubule-dependent (Qualmann and Kessels, 2002). The following step after internalization is delivery to the early endosomes (EE), that are vesiculo-tubular organelles, identified by resident membrane protein EEA1 and associated small Rho family GTPase Rab5, usually located in the peripheral parts of the cell having the ability to go through homotypic fusion with other EEs. From there the cargo is sorted either to the degradative pathway to the lysosomes or recycled back to the plasma membrane directly or through recycling endosomes identified by resident Rabs 5, 4 and 11. Cargo entering the recycling pathway consists mainly of housekeeping receptors such as transferrin receptor (TfR) and low density lipoprotein receptor (LDLR). Also transport to the TGN from EEs is possible and these cargoes, together with newly synthesized proteins, can exit the TGN and traffic either to the plasma membrane or to late endosomes, and to a minor extent to early endosomes. Cargo in the degradative pathway (for example low density lipoprotein LDL and EGFR) is transported form the EEs multivesicular carrier vesicles (ECV/MVBs) that acquire their internal membrane profiles as a result of the inward invagination of the delimiting membrane containing enriched phosphatidylinositol 3-phosphate (PI3P) and cholesterol. Ubiquitinylation of receptors, destined for downregulation, targets them for sorting into multivesicular bodies. ECV/MVBs fuse with late endosomes enriched in lysobisphosphatidic acid (LBPA) and associated Rab7 and Rab9. Cargo, such mannose 6-phosphate receptors (M6PRs), can be are recycled back to the TGN from late endosomes. Upon fusion with the lysosome, cargo is degraded. (Perrais and Merrifield, 2005; Soldati and Schliwa, 2006; van der Goot and Gruenberg, 2006). The lysosome is a highly acidic organelle having a delimiting

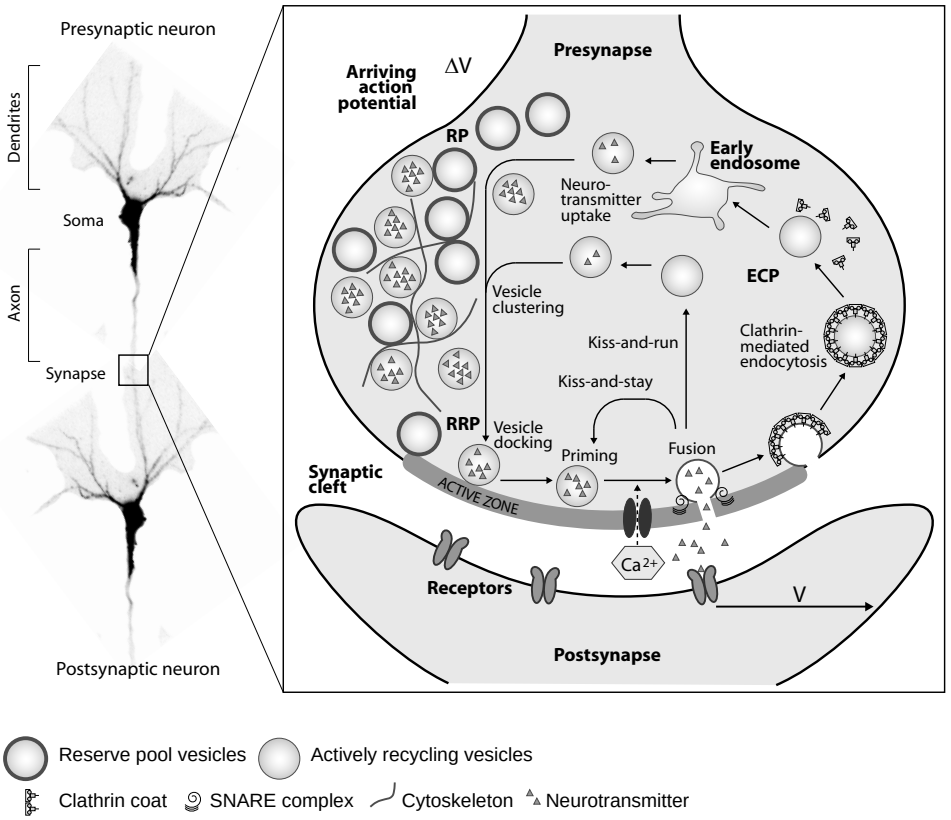
membrane and contains large numbers of lysosomal hydrolases. Lysosomal acidification is achieved through the V-type H^+ -ATPase complex that powered by ATP translocates protons. Over half of the lysosomal membrane mass is composed of membrane proteins LAMP-1, LAMP-2, LIMP-1 and LIMP-2 (Kornfeld and Mellman, 1989). Lysosomes also lack the cation-dependent (46kDa MPR) and cation independent (300kDa CI-MPR) mannose 6-phosphate receptors. Lysosomes have various functions including down-regulation of cell surface receptors, release of nutrients from endocytosed material, turnover of cellular proteins, repair of membranes, inactivation of pathogens, loading antigens for presentation onto MHC class II molecules in antigen presenting cells (Eskelinen, et al. 2003).

1.5 Membrane trafficking in neurons and synaptic transmission

The nerve cells generate electrical signals to convey information. These signals are transmitted to other cells via synaptic connections. Neurotransmission is dependent of the electrical potential of the cell membrane, and neurotransmission is mediated by the action potential. The action potential is initiated at the cell body and travels to the presynaptic nerve terminal. The action potential induced transient increase in Na^+ permeability results in membrane depolarisation and the opening of Ca^{2+} channels at the presynaptic membrane. The calcium influx triggers fusion of the synaptic vesicles (SVs) to the presynaptic membrane and neurotransmitter is released. Neurotransmitters traverse the synaptic cleft and bind to their respective receptors on the postsynaptic membrane and transfer the signal to the postsynaptic neuron. At the presynaptic neuron, excess free calcium is sequestered intracellularly or removed to the extracellular space and transient rise in K^+ permeability repolarizes the neuronal membrane reconstituting the resting state. (Südhof, 2004)

Neurotransmission depends on the regulated release of transmitter molecules form specialized secretory vesicles in the synapse. The packaging of cargo into these secretory vesicles is carried out by vesicular transporters and the activity of the transporters affects levels of neurotransmitter available thereby affecting synaptic function. The transporters all function by a same principle: a vacuolar proton pump driven with ATP creates an electrochemical gradient enabling active transport of neurotransmitters into

Figure 2. Synaptic Membrane Transport Pathways.



The synaptic vesicles are arranged into different functional pools. The docked pool or the pool of actively recycling vesicles (RRP) are mobilized upon action potential arrival. Reserve pool (RP) and endocytosis/ exocytosis cycling pool (ECP) replenish the readily relesable pool. The vesicles are loaded with neurotransmitter and dock at the active zone. The vesicles are primed making them competent for Ca^{2+} triggered fusion. Arriving action potential depolarizes the presynaptic plasma membrane and triggers Ca^{2+} influx through channels. Incoming Ca^{2+} triggers SNARE mediated fusion and neurotransmitter is released. Upon binding to postsynaptic receptors it induces depolarization of the postsynaptic membrane. At the presynapse vesicles are recycled through the fast route by kiss-and-stay or kiss-and-run recycling or by the slower clathrin-mediated endosomal pathway.

Adapted from: Schweizer and Ryan, 2006; Südhof, 2004

SVs. Each neurotransmitter group has their own transporters. (Ahnert-Hilger, et al. 2003) Synaptic vesicles are clustered to the presynaptic active zone by scaffolding proteins aided by several adaptors. These processes are described in detail in chapter:

Presynaptic terminal differentiation, transport and assembly. The SVs are arranged into different functional pools consisting of varying types and number of vesicles depending on neuron type (Rizzoli and Betz, 2005). The docked pool or the pool of vesicles that is drawn from by the initial action potential is referred to as the readily-releasable pool (RRP) (Saviane and Silver, 2006). It is replenished from the recycling or reserve pool (RP), also called the endo/exo (endocytosis and exocytosis) cycling pool (ECP) (Saviane and Silver, 2006). The remaining vesicles are grouped into the non-recycling pool or the cycling-dead pool (Rizzoli and Betz, 2005). In resting nerve terminals, synaptic vesicle pools are tethered in place by a a network of filaments and cytoskeletal components. Stimulation is proposed to increase vesicle mobility for controlled fusion events via synapsins that form a link between the SVs and the actin cytoskeleton and are activated by calcium (Llinas, et al. 1985). The vesicles are primed making them competent for Ca^{2+} triggered fusion. Vesicle priming, the extent of SNARE (soluble N-ethylmaleimide sensitive fusion (NSF) attachment protein receptor) fusion complex formation and/or the distance to the next calcium channel affect the fusion probability of a given docked vesicle (Schweizer and Ryan, 2006). The fusion SNARE complex drives the fusion of the trafficking synaptic vesicle membrane to the target membrane following target recognition. The complex forming proteins are highly conserved throughout eukaryotic evolution and function in most membrane fusion events. 36 members of the SNARE superfamily are present in humans. (Bonifacino and Glick, 2004; Jahn and Scheller, 2006) The SNARE proteins function in related pairs, with one partner being localized on the vesicle and the other set on the target membrane. Correct assembly of the SNARE pair is also involved in establishing fusion specificity. The SNARE driven membrane fusion is powered by free energy that is released during the formation of a four-helix bundle of the SNARE complex. The establishment of the bundle, or *trans*-SNARE complex, leads to a tight connection of the fusing membranes, and initiates the membrane merger. Upon membrane fusion, the SNAREs are present on the same membrane in a *cis*-complex. The SNARE proteins are recycled for repeated cycles of membrane fusion and the recycling is achieved through the dissociation of the helical bundle by the AAA+ protein NSF (N-ethylmaleimide-sensitive factor) and SNAP (soluble NSF-attachment proteins)(Jahn and Scheller, 2006). Neuronal SNAREs include Synaptotagmins (Syt): Syt1 is required for fast Ca^{2+} mediated exocytosis, whereas Syt3, 6 and 7 are implicated in slow asynchronous release (Sugita, et al. 2002). Synaptic vesicles also have

their own Rab proteins (Rab3A-D, Rab5 and Rab11), that mediate membrane attachment via effector molecules.

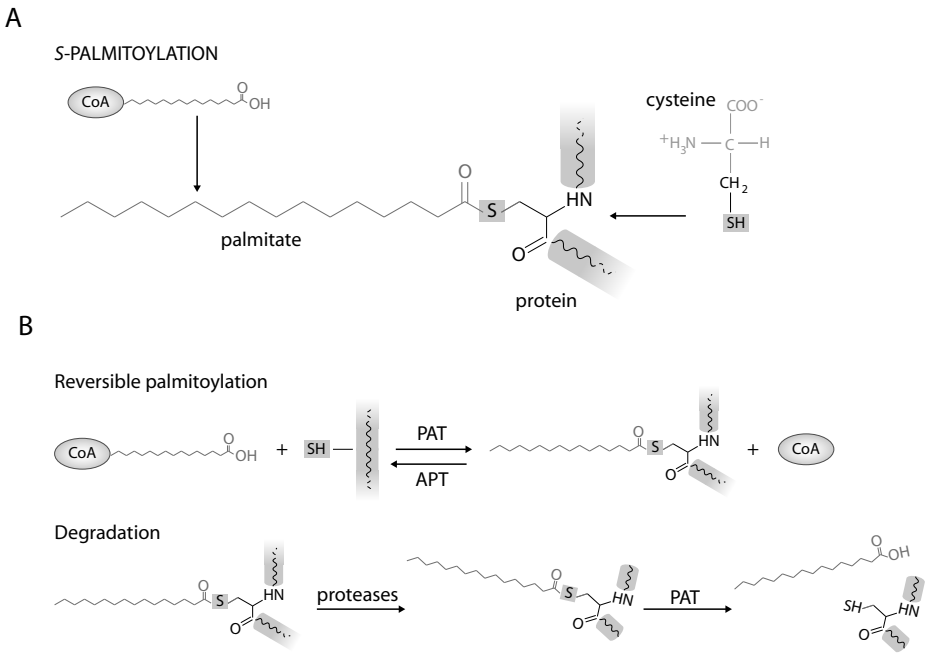
Upon fusion of the vesicles and release of neurotransmitters the SVs are endocytosed and locally recycled for another round of employment within nerve terminals. It is not fully understood how the exocytic process triggers endocytosis but two different mechanisms have been suggested: SV proteins appearing on the PM at exocytosis drive their own retrieval through adaptor-recognition of retrieval motifs on the cytoplasmic tails of the proteins or elevation in intracellular calcium by the action potential initiating exocytosis activates the endocytic machinery. The endocytosis is carried out through alternative routes: 1) slow clathrin mediated direct- or intermediate utilizing endosomal recycling 2) fast direct kiss-and-stay recycling or kiss-and-run recycling through the reserve pool. (Schweizer and Ryan, 2006; Südhof, 2004)

1.6 Protein palmitoylation: regulator of function and trafficking

Protein palmitoylation, the addition and removal of the fatty acid palmitate to proteins, has a major role in controlling and modulating protein-membrane interactions, protein trafficking, and enzymatic activity (Huang and El-Husseini, 2005; Linder and Deschenes, 2007). Palmitoyltransferases that add palmitate to proteins were first identifed in the yeast *Saccharomyces cerevisiae* and *Drosophila* fly (Linder and Deschenes, 2003). Several palmitoyltransferases have since then also been identified in mammalian systems. These include mouse and human DHHC proteins, sharing a cysteine rich Asp-His-His-Cys (DHHC) motif (Fukata, et al. 2004; Huang, et al. 2004). While mammalian palmitoylating DHHC proteins have been identified in the ER, Golgi and the plasma membrane data is still limited on the identity as well as localization of depalmitoylating thioesterases. To date only two palmitate removing enzymes have been identified. The acylprotein thioesterase APT1 resides in the cytosol and removes palmitate from membrane associated proteins (Duncan and Gilman, 1998). The other palmitoyl removing enzyme is palmitoyl protein thioesterase 1 (PPT1). It is the only enzyme described to date that depalmitoylates proteins destined for degradation in the lysosomes (Lu and Hofmann, 2006). PPT1 has possible additional roles in the neurons because a wide range of proteins with different characteristics are palmitoylated. Therefore it is likely that multiple substrate specific acyltransferases and thioesterases

act in multiple subcellular compartments. (Linder and Deschenes, 2003; Linder and Deschenes, 2007). A schematic representation of the protein palmitoylation/depalmitoylation cycle is presented in Figure 3.

Figure 3. Protein Palmitoylation



Schematic representation of palmitoylation of cellular proteins.

A) The 16-carbon lipid, palmitate, is attached to the thiol group (SH) of a cystein amino acid residue.

B) The addition of palmitate from coenzyme A (CoA) to the target protein is catalyzed by protein acyltransferases (PAT). The removal of palmitate is mediated by acylprotein thioesterases (APT). Palmitoylated proteins destined for degradation in the lysosomes are cleaved into peptides by various proteases from which palmitate is consequently removed.

1.6.1 Reversible palmitoylation and dual lipid modifications

Protein palmitoylation is a reversible modification that allows dynamic regulation of protein function. Palmitoylation or S-acylation is a post-translational modification in which a 16 carbon saturated fatty acid palmitate is incorporated into the side chain of cysteine residue of a newly synthesized protein post-translationally. Proteins that are

palmitoylated often also carry additional lipid modifications. For example signal-transduction proteins, such as guanine-nucleotide-binding G protein subunits and non-receptor tyrosine kinases, are reversibly modified with a co-translational N-myristoylation followed by post-translational palmitoylation. (Resh, 1999). The Ras superfamily small GTPases are also reversibly modified but have a more complex series of post-translational modifications leading to prenylation, a farnesyl or a geranylgeranyl isoprenoid modification, followed by palmitoylation (Schafer and Rine, 1992; Zhang and Casey, 1996). The Hedgehog family proteins have, in contrast to the aforementioned, irreversible dual-lipid modifications with cholesterol at the C-terminus and palmitate in the N-terminus (Mann and Beachy, 2004; Miura and Treisman, 2006). The thioester bond formed in S-acylation is labile enabling palmitate removal (Magee and Courtneidge, 1985; McIlhinney, et al. 1985). Depalmitoylation, or removal of the palmitate moiety, occurs when an enzyme hydrolyses the thioester bond between the carboxyl end of the fatty acid and the sulfhydryl group of the cysteine residue. The palmitate turnover can be constitutive or regulated (Smotrys and Linder, 2004).

1.6.2 Cellular targets of palmitoylation

Both integral and peripheral membrane proteins can be palmitoylated. Palmitoylation renders the target protein virtually always membrane bound, whereas other fatty acid modifications, such as prenylation and myristoylation, target proteins to the cytoplasm as well as to the membranes (Casey, 1995). A vast number of proteins are known to be palmitoylated and the palmitoylproteome, or total set of known and predicted palmitoylated proteins in a cell, has grown extensively in recent years. The classical targets of palmitoylation include G-protein subunits and G-protein coupled receptors (Dunphy and Linder, 1998), Src family protein tyrosine kinases (Milligan, et al. 1995), acetycholinesterase (Roberts, et al. 1988), nitric oxide synthase (Robinson, et al. 1995) and the transferrin receptor (Jing and Trowbridge, 1987). Recent additions comprise SNAREs, kinases, protein phospatases, yeast vacuole proteins and amino acid permeases (reviewed in Linder and Deschenes, 2007). Moreover, palmitoylation is remarkably ubiquitous in the nervous system. Palmitoylated proteins in neurons include growth-associated protein-43 (GAP-43), synaptosomal protein SNAP-25, the postsynaptic density protein PSD-95, AMPA- and GABA receptor subunits (Hayashi, et al. 2005; Keller, et al. 2004; Lu and Hofmann, 2006). The synaptic vesicles contain

all the components for palmitoylation of SNARE proteins, however the exact sites where palmitoylation and depalmitoylation occur in cells are still unclear. The neuronal SNARE SNAP-25 (25-kDa synaptosome-associated protein), has two SNARE motifs joined by a palmitoylated linker. Also SNAREs that have transmembrane domains can be palmitoylated. This has been shown to protect SNAREs from ubiquitin-pathway mediated degradation (Valdez-Taubas and Pelham, 2005).

1.6.3 Palmitoylation in regulating intracellular traffic

Palmitoylation regulates transport of proteins between organelles, targeting of proteins into the nerve terminals, traffic in the terminals and also clustering of proteins in specific membrane compartments (Linder and Deschenes, 2007). One of the best characterized examples of intracellular targeting by palmitoylation are the Ras superfamily small GTPases. H-Ras, carrying two palmitoylation sites, and N-Ras carrying one palmitoylation site, reside on the plasma membrane, Golgi or ER depending on their palmitoylation statuses (Roy, et al. 2005). Moreover, their palmitoylation statuses also affect their membrane microdomain localization on the membrane in question: A single palmitoylation of H-Ras on Cys184 results primarily in Golgi localization. However, the fraction residing on the plasma membrane undergoes GTP-regulated lateral diffusion between cholesterol rich microdomains/lipid rafts and cholesterol-independent microdomains, similar to dually palmitoylated H-Ras. By contrast, single palmitoylation of H-Ras on Cys181 leads to efficient plasma membrane localization, but this form of H-Ras localizes solely to lipid rafts. In this way, the Cys181 palmitoylated form of H-Ras behaves like N-Ras (Roy, et al. 2005).

2. Neuronal maturation

2.1 Establishment and maintenance of neuronal polarity

Mature neurons are highly polarized cells that have the ability to transmit intercellular signals from one compartment to another. Most neurons have a single axon and several dendrites and the axonal and somatodendritic compartments are formed during neuronal maturation. The different cellular compartments aquire distinct structural

and molecular properties guided by several polarity regulating molecules. Cultured hippocampal neurons develop and maintain a single axon and several dendrites and they have been used extensively to characterize the molecular mechanims underlying neuronal polarization. *In vitro* maturation of these neurons takes about seven days and is divided into five stages: 1) After plating the neurons form a protrusion veil and few spikes. 2) Spikes are transformed into short neurites of equal length. 3) One neurite starts growing rapidly forming an axon and establishing neuronal polarity. 4) Remaining protrusions aquire dendritic characteristics. 5) Neurons establish synaptic contacts forming a network (Reviewed in Craig and Banker, 1994). Neurons have an intrinsic polarization program but also extracellular cues and their downstream signal regulate polarization in physiological surroundings. Recent evidence suggests that these are regulated by PI 3-kinase, Rho family GTPases, cytoskeleton related proteins and the Par complex (Arimura and Kaibuchi, 2005).

2.1.1 Axon specification

Several mechanisms have been proposed to trigger the initial event of neuronal polarization, or axon specification. These include exclusion of polyribosomes from the growing axon and higher concentration of intracellular membranes in the axonal growth cone (Deitch and Banker, 1993) or the localization of axon specific proteins such as GAP-43 (Goslin, et al. 1988). Regulating microtubule assembly, the endocytosis of adhesion molecules, and the reorganization of actin filaments are key events in axon elongation. The centrosome and directional microtubule assembly have been shown to guide the early neuronal polarization. In cultured hippocampal neurons, the centrosome, Golgi apparatus, and endosomes cluster together to the area where the axon will form and prevention of the centrosome functions inhibit neurite formation (de Anda, et al. 2005). However, this effect may rather result from the disturbance of the centrosome controllers, microtubules, dynein and PI 3-kinase- and Cdc42-mediated signals and not the centrosome position *per se* (Arimura and Kaibuchi, 2005). PI 3-kinase and its lipid product [PI(3,4,5)P3; PIP3] or PIP3 are essential for neuronal polarization. PI 3-kinase is specifically activated at the tip of the future axon and application of a PI 3-kinase inhibitor totally prevents axon formation (Shi, et al. 2003). Axon elongation requires rearrangement of the cytoskeletal components, the actin filaments, intermediate filaments and microtubules, which are regulated by Rho

family GTPases. Dynamic changes in actin filaments in the tip of the growing axon, allowing microtubule growth into distal parts of the growth cone, have been implicated (Bradke and Dotti, 1999). Putative Rho family GTPases for axon specification includes Cdc42 (cell division cycle 42), Rac1, and RhoA (reviewed in Govek, et al. 2005). PI 3-kinase activity is also required for proper localization of Cdc42 and the Par-kinesin polarity complex at tips of the axon (Nishimura, et al. 2005; Shi, et al. 2003). During axon elongation vesicles are targeted to the growth cone to promote neurite outgrowth and synaptogenesis. The secretory pathway exocyst complex is an essential macromolecule in this process. It consists of eight subunits which interact in a polar fashion with the target membrane, Rab GTPases associated with secretory vesicles and the cytoskeleton, to mediate vesicle fusion (Vega and Hsu, 2001).

2.1.2 Polarized transport and targeting of neuronal traffic

Neurons must retain their polarity throughout their lifespan in order to effectively convey intercellular messages across the cell. Intracellular trafficking is important not only to the establishment but also the maintenance of neuronal polarity. However, neuronal trafficking mechanisms are still relatively poorly understood. The major regulator seems to be targeting of transport by the specific organization of microtubules in the neurons. In the axon the microtubules are organized in only one orientation with the plus end of the tubule facing the distal part of the axon, whereas in dendrites the microtubules are in a mixed orientation (Baas, et al. 1988; Burton and Paige, 1981). Proteins and cargo containing vesicles are selectively transported from the soma to the neuronal extensions along the microtubules utilizing kinesin and dynein family motor proteins (reviewed in Hirokawa and Takemura, 2005). The transport from the cell body towards the extensions is mainly mediated by kinesin superfamily proteins (KIFs) (Aizawa, et al. 1992), whereas dynein superfamily proteins carry out retrograde transport towards the soma (Harada, et al. 1998). Proteins regulating neuronal polarity are specifically transported to the growing axon. For example Par3 is known to be transported to the tip of the axon by Kinesin-2 through direct interaction with KIF3A (Nishimura, et al. 2004). Some KIFs are always dendritic, for example the plus end directed KIF21B (Marszalek, et al. 1999) whereas others such as KIF21A and KIF5 (Marszalek, et al. 1999; Nakata and Hirokawa, 2003) are axonal. This differential distribution suggests that KIFs are able to recognize the microtubule population in the axonal initial segment, but the molecular

mechanisms underlying this function are currently not understood (Winckler, 2004). In addition to the KIF mediated axonal targeting also other mechanisms have been identified. Sorting at the TGN level has been implicated: Some axonal cargoes, for example the presynaptic protein synaptophysin, are sorted to axonal cargo-enriched transport carriers vesicles in the trans-Golgi network and are transported anterogradely along the axon in distinct post-Golgi carriers (Kaether, et al. 2000). Also preferential domain specific fusion involving SNARE complexes and their regulators may serve as targeting mediators (Foletti, et al. 1999; Gerst, 2003).

Palmitoylation acts as a targeting signal to axons for growth associated protein 43 (GAP-43), paralemmin (Kutzleb et al. 1998) and glutamate decarboxylase GAD65 (Kanaani, et al. 2002; Kanaani, et al. 2004; Kutzleb, et al. 1998). Palmitoylation is also a key factor in neuronal development. Palmitoylation affects dendritic spine formation via paralemmin aided with Rho family GTPases cdc-42 and ARF6 (Gauthier-Campbell, et al. 2004). It also regulates axonal pathfinding, synapse formation and growth-cone maturation via adhesion molecules NCAM140, DCC (axon guidance receptor deleted in colorectal cancer) and growth associated protein GAP-43 and post-synaptic receptor and ion channel clustering (Christopherson, et al. 2003; Craven, et al. 1999; Herincs, et al. 2005; Kim, et al. 2004; Niethammer, et al. 2002).

Endocytic processes play a role in axon outgrowth and targeting of intracellular traffic in neurons. During axon outgrowth, new membrane and associated proteins are inserted in the growing tip using molecular machinery distinct from synaptic vesicle fusion machinery (Futerman and Banker, 1996; Martinez-Arca, et al. 2000). For example, endocytic proteins syntaxin13 and VAMP7 are required for axon outgrowth (Alberts and Galli, 2003; Alberts, et al. 2003; Hirling, et al. 2000). Their effect is postulated to be local in growth-cone associated endosomes controlling adhesion-detachment cycles (Alberts, et al. 2003). Another option is that endocytosed material is in fact receptors that have ‘leaked’ into the incorrect domain and are transported from the somatodendritic compartment to the axon and this transport is regulated by syntaxin and VAMP7 (Winckler, 2004). Transcytosis has also been described in neurons: Neurotrophins are endocytosed at axonal sites and transported back to the cell body for participation in cellular signaling events (Barker, et al. 2002; Ginty and Segal, 2002).

Conversely, transferrin and polymeric Ig receptor (pIgR) travel from the dendrites to the axon (de Hoop, et al. 1995; Hemar, et al. 1997; Jareb and Banker, 1998).

Many factors affect intracellular targeting and polarization in neurons. Particularly axonal targeting seems to be a complex multi-step process with various degrees of selectivity at different steps and also corrective functions. Even though the physiological function and relevance of these steps still remains elusive, modulation of pathway choices would allow dynamic changes in cargo targeting and moreover neuronal function.

2.2 Presynaptic terminal differentiation, transport and assembly

During neuronal maturation synapses are assembled into a highly differentiated and organized structure containing the presynaptic terminal and corresponding postsynaptic part. The major events in presynaptic terminal differentiation are the formation of the active zone and the clustering of synaptic vesicles. The active zone is formed by closely packed synaptic vesicles near the presynaptic plasma membrane opposite to the postsynaptic density. In mature chemical synapses the synaptic vesicles mediate neurotransmitter release and are recycled in the area flanking the active zone. In this perisynaptic zone, synaptic vesicles that have undergone fusion are retrieved by clathrin-mediated endocytosis (Gundelfinger, et al. 2003). The three main complexes enabling release of contents and recycling of synaptic vesicles are 1) the core SNARE which mediates vesicle docking and fusion 2) the exocytosis complex including Munc18/UNC-, Munc13/UNC-13, and synaptotagmin and 3) the complex of variable contents that tethers and organizes vesicles and endo/exocytosis machineries in the active zone. These complexes are explained in detail in the previous chapter describing the synaptic vesicle cycle. Associated with the function of these complexes are also the cytoskeletal proteins, scaffolding proteins, voltage gated calcium channels and cell adhesion molecules. Interactions between these main players create a network that organizes the structure and function of the presynaptic terminal.

2.2.1 The active zone components

To date, five active zone cytomatrix (CAZ) enriched protein families have been identified and functionally analyzed: UNC13/Munc13 proteins, RIMs (Rab3-interacting molecule), Bassoon and Piccolo, ELKS (ERC/CAST), and liprins-α (Schoch and Gundelf-

inger, 2006). Through specific interactions, these proteins form a scaffold at the active zone and organize the release and retrieval of synaptic vesicles and regulate changes in neurotransmitter release in short-term and long-term forms of synaptic plasticity.

At the active zone two major proteins are responsible for scaffolding in the presynaptic cytomatrix (CAZ): Bassoon and Piccolo are among the earliest protein components present at the developing presynapses. (Garner, et al. 2000). The active zones are pre-assembled in neuronal cell bodies and transported as so-called Piccolo-Bassoon transport vesicles (PTVs) to sites of synaptogenesis (Antonova, et al. 2001; Fejtova and Gundelfinger, 2006;). Also AZ proteins such as Syntaxin, SNAP25, and N-cadherin, are carried along axons in PTVs (Zhai, et al. 2001). Synaptic assembly is initiated by local trapping or maturation of different presynaptic components following synaptic target recognition (Ahmari, et al. 2000; Shapira, et al. 2003). In addition to stable components of the active zone various components undergo redistribution with synaptic activity (Tao-Cheng, 2006). RIMs (Rab3-interacting molecule family) mediate the insertion of PTV contents to the plasma membrane (Dulubova, et al. 2005; Shapira, et al. 2003). RIMs can also interact with other multiple synaptic proteins for example ELKS, Piccolo, synaptotagmin, SNAP25, Liprin-α and N-type voltage-dependent calcium channels (reviewed in Schoch and Gundelfinger, 2006). In mammals ELKS also called CAST (CAZ-associated structural protein) proteins are required for correct localization of RIM proteins at the active zone, calcium channel clustering and vesicle release (Kaufmann, et al. 2002; Kittel, et al. 2006).

The highly regulated distribution of active zones in developing neurons is crucial for the correct function of neuronal circuits. The development of neuronal arborizations must include mechanisms to regulate key features such as the spacing of terminals, the frequency of branching, and the distribution and density of release sites. Yet, many of these regulatory mechanisms and their specific features are still poorly understood. Endocytic events have been suggested to have a role in the controlling of these events. Evidence in several *Drosophila* endocytosis mutants shows that impaired endocytosis causes formation of extra satellite synapses. The exact mechanism behind this is unknown but several hypotheses exist on the underlying reasons: Extra boutons may be an indirect consequence of accumulation of excess surface membrane. Also possible involvement of endocytic proteins in cytoskeletal arrangements has been suggested (Dickman, et al. 2006).

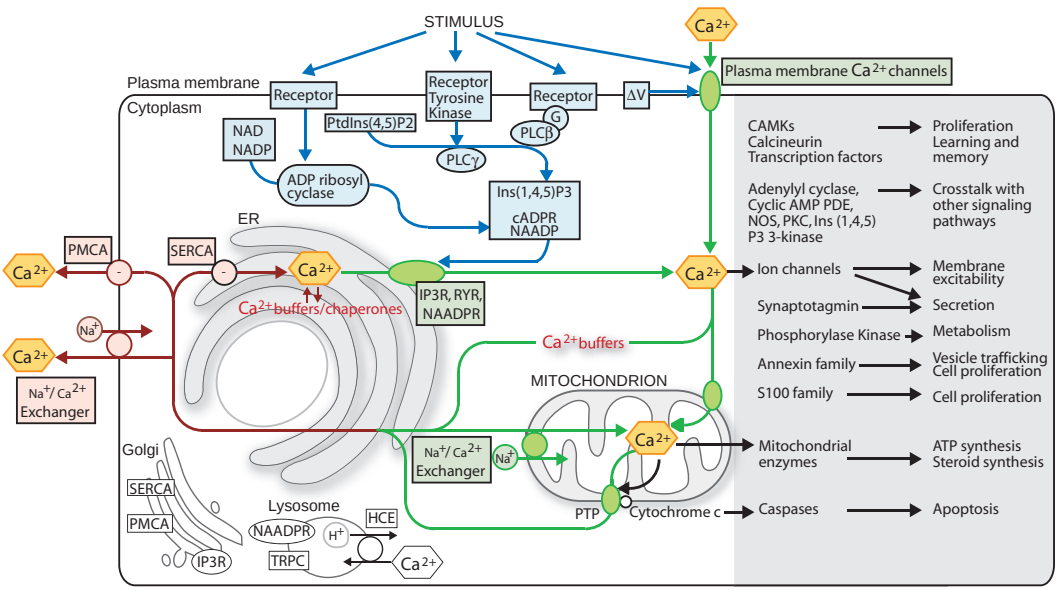
2.3 Calcium metabolism and neuronal maturation

The calcium ion is a ubiquitously employed intracellular messenger. It is a key regulator of central cellular events in development, neuronal function, secretion and controlled cell death. Exceeding concentrations of calcium are toxic to cells and too small concentration causes cellular dysfunction and therefore the amount of intracellular free calcium needs to be tightly regulated. Cells regulate their intracellular calcium concentrations within a narrow physiological range aided by an array of cell surface and intracellular receptors, channels, pumps, and organelles (reviewed in Berridge, et al. 2000). In addition, especially different neuron types utilize various calcium binding proteins to buffer changes in intracellular calcium (Schwaller, et al. 2002). Elements of intracellular calcium signalling are described in figure 4.

2.3.1 Calcium signalling in the formation of synapses

Neuronal activity and communication with neighbouring cells influences neuronal development, and even though signalling mechanisms that link activity to morphology are not well understood, it is evident that calcium plays a key role. In developing neurons initial synaptic contact leading to signalling events results in elevation of intracellular calcium levels. This is achieved through influx of extracellular calcium or release of calcium from intracellular stores. Activity induced fluctuations in intracellular calcium levels result in changes in neuronal morphology through immediate local mechanisms and more prolonged mechanisms acting on the transcriptional level. Fast local responses are mediated by calcium sensitive signalling proteins and result in restructuring of the dendritic cytoskeleton, affecting spine dynamics and formation, initiation of filopodia, branch stability and local translation. (Maletic-Savatic, et al. 1999; Sutton, et al. 2004; Wong and Ghosh, 2002; Wu, et al. 2001). Strict temporal regulation of calcium signalling and its downstream components has been suggested to have an important role in the developmental program (Gruol, et al. 2006). Time-dependent changes in intracellular Ca^{2+} concentrations are important regulators of synapse formation (Berridge, et al. 2000; Crabtree, 2001). In neuronal growth cones, Ca^{2+} waves are spatially and temporally highly regulated during growth-cone turning, and their elimination inhibits normal neuronal differentiation. Ca^{2+} links external stimuli to internal processes such as growth-cone protrusion, axonal path finding and initial

Figure 4. Elements of Cellular Ca^{2+} Signalling.



Ca^{2+}-mobilizing signals (blue) are mediated by variety of cell-surface receptors, including G-protein (G)-linked receptors and receptor tyrosine kinases, in response to different stimuli. Generated signals are as follows:
a) inositol-1,4,5-trisphosphate (Ins(1,4,5)P3) is hydrolysed from phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) by a family of phospholipase C enzymes (PLC , PLC)
b) cyclic ADP ribose (cADPR) and nicotinic acid dinucleotide phosphate (NAADP), both derived from nicotinamide-adenine dinucleotide (NAD) and its phosphorylated derivative NADP by ADP ribosyl cyclase

Intracellular calcium concentration increasing mechanisms (in green) include plasma membrane Ca^{2+} channels responding to transmitters or to membrane depolarization (V), and intracellular membrane Ca^{2+} channels — the Ins(1,4,5)P3 receptor (IP3R), ryanodine receptor (R YR), NAADP receptor responding to ligands respectively. The Ca^{2+} flowing into the cytoplasm activates different Ca^{2+} sensors in mitochondria and internal membranes and the plasma membrane, which initiate a variety of Ca^{2+} -sensitive cellular processes (black arrows), depending on cell type and context. Ca^{2+} restoring back to resting level is achieved by mechanisms that move Ca^{2+} out of the cytoplasm (in red): the Na^{+} /Ca^{2+}exchanger and the plasma membrane Ca^{2+}ATPase (PMCA) pumps Ca2+ out of the cell and the endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps it back into the ER. In addition intracellular Ca^{2+} is regulated by Golgi and lysosome resident components.

(CAMK, Ca^{2+}/calmodulin-dependent protein kinase; cyclic AMP PDE, cyclic AMP phosphodiesterase; NOS, nitric oxide synthase; PKC, protein kinase C; PTP, permeability transition pore; TRPC, transient receptor potential cation channel related Ca^{2+} channel mucolipin-1; HCE, lysosome resident un-characterised H^{+}– Ca^{2+} exchanger that utilizes a proton gradient.

Adapted from
Berridge, M.J., Lipp, P. & Bootman, M.D. The versatility and universality of calcium signalling.
Nat. Rev. Mol. Cell. Biol. 1, 11–21 (2000).

formation of synaptic contacts (Gomez and Spitzer, 1999; Gomez, et al. 2001; Spitzer, et al. 2000). The ion channels that regulate these processes in the developing brain

are poorly characterized. The best characterized are mammalian transient receptor potential cation channels (TRPCs) (Minke, 2006). The TRPC family comprises seven different plasma membrane channels (TRPC1–TRPC7) (Minke, 2006; Pedersen, et al. 2005) of which TRPC family member 5 (TRPC5) is important in controlling neurite extension and growth cone morphology. It is a part of a larger precursor active zone complex and travel to the forming synapse in cytoplasmic transport packets together with other developmentally regulated proteins (Greka, et al. 2003).

2.3.2 Calcium binding proteins and neuronal identity

Specific sub-populations of neurons in the CNS can be identified by calcium binding proteins they express. Differential distribution reflects specific functional characteristics of different calcium binding proteins and also needs of different neuron types (Baimbridge, et al. 1992). The calcium binding proteins have varied affinities and kinetics. The best characterized family includes calbindin D-28K/calbindin1, calretinin and parvalbumin, that have structural similarities the first two having fast kinetics and the latter slow kinetics respectively (Lee, 2001; Schwaller, et al. 2002). Expression of these proteins has a strong developmental correlation: calbindin D-28K/calbindin1 appears at the same time in developing neurons as voltage-gated calcium channels start to emerge and calretinin expression is temporally correlated with the onset of synaptic inputs respectively (Hack, et al. 2000). Expression of both proteins is also parallel to maturation in neuronal circuits (Alcantara, et al. 1996; Schierle, et al. 1997; Schlosser, et al. 1999). Parvalbumin in contrast is expressed highly in later stages (Baimbridge, et al. 1992; Hof, et al. 1999). The developmental significance of these proteins is thought to also be manifested through their neuroprotective actions. Especially calbindin D-28K/calbindin1 expression protects cells from activity dependent glutamate induced perturbations in calcium homeostasis (Mattson, et al. 1991). In addition to developmental significance calcium binding proteins are central in maintenance of calcium homeostasis in mature neuronal function. The dependence between calcium binding proteins and calcium itself is thought to be two-way: Changes in calbindin D-28K/calbindin1 expression alter calcium homeostasis (Lledo, et al. 1992). Changes in calcium concentration, such as chelation of extracellular calcium, and blocking of voltage-operated calcium channels, inhibit upregulation of expression (Boukhaddaoui, et al. 2000).

2.3.3 Regulating intracellular calcium levels

Neurons have many mechanisms to regulate their calcium in addition to direct free calcium binding. The neuronal calcium release/flow into the cytoplasm is regulated through the plasma membrane and intracellular membrane responses. On the plasma membrane Ca^{2+} channels in different parts of the cell carry out separate functions. N- and P/Q-type voltage gated or voltage operated calcium channels (VOCs) are located at axonal synaptic endings and trigger the release of neurotransmitters. The L-type VOCs on the cell body and proximal dendrites supply the Ca2+ signals for inducing gene activation and transcriptional modulation and respond to the small depolarizations at synaptic spines (Evans and Zamponi, 2006; Mermelstein, et al. 2000). The Ca^{2+} signals in spines are also responsible for mediating the early synaptic modifications important in learning and memory. They are mediated by calcium entry through VOCs and receptor operated channels (such as NMDA (N-methyl-D-aspartate) receptors) and by release from internal stores through channels such as inositol-1,4,5-trisphosphate receptors (InsP3R) or ryanodine receptors (RYR) (reviewed in Berridge 1998). In memory and learning the role of InsP3Rs is correlation of pre- and postsynaptic inputs through coincidence detection. InsP3 receptors respond to Ca^{2+} flowing through VOCs in response to membrane depolarization and simultaneous appearance of Ins(1,4,5)P 3 produced by metabotropic glutamate receptors (mGluR1). The result is synergistic release of calcium from the ER internal stores (Choe and Ehrlich, 2006). InsP3Rs and ryanodine receptors are also central in Ca^{2+}-induced Ca^{2+} release in which cytoplasmic Ca^{2+} activates the release of Ca^{2+} from internal stores through an autocatalytic mechanism. Furthermore, calcium controls the transcription of its own transporters and channels in different domains of developing neurons (Carafoli, et al. 1999).

2.3.4 Organelles involved in the maintenance of calcium homeostasis

High calcium concentration is toxic to neurons, it has to be removed rapidly after it has completed its signalling functions. For this cells have several mechanisms: ion pumps and exchangers remove excess Ca^{2+} and it is also sequestered into the mitochondria and other cellular organelles (Michelangeli, et al. 2005; Prinsen, et al. 2002; Uehara, et al. 2005). The plasma membrane Ca^{2+}-ATPase (PMCA) pumps and

Na^+/Ca^{2+} exchangers extrude Ca^{2+} to the outside and the sarco-endoplasmic reticulum ATPase (SERCA) pumps return Ca^{2+} to the internal stores.

Another key component in calcium metabolism is the mitochondrion. It sequesters calcium rapidly and has a high accumulation capacity while the mitochondrial matrix contains buffers that efficiently prevent excess calcium build-up. The mitochondria sequester Ca^{2+} utilizing the mitochondrial Ca^{2+} uniporter (MCU) located on the inner mitochondrial membrane (Bernardi, 1999; Gunter, et al. 2004). Mitochondria extrude protons to create the electrochemical gradient necessary for ATP synthesis and this gradient is also used to drive Ca^{2+} uptake through the MCU. The high levels of cytosolic Ca^{2+} needed to activate the MCU exist in localized micro-domains close to clusters of ER or plasma membrane Ca^{2+} channels and mitochondria accumulate Ca^{2+} more effectively when they are close to Ca^{2+}-releasing channels (Dyer, et al. 2003; Rizzuto, et al. 1993; Rizzuto, et al. 2004). Mitochondria also regulate ion channels that open in response to binding of an extracellular ligand (so called store-operated Ca2+ entry) (Glitsch, et al. 2002). Various proteins implicated in neurodegeneration, such as presenilins, and apoptosis regulatory proteins modulate mitochondria and ER interplay and calcium handling. Once the cytosolic Ca^{2+} has returned to its resting level, the mitochondrion decreases its Ca2+ concentration by pumping excess calcium into the cytoplasm through the mitochondrial Na^+/Ca^{2+} exchanger (NCE) or the permeability transition pore (PTP) (Bernardi, 1999). The PTP also contributes to the initiation of apoptosis during the occurrence of irreversibly high Ca^{2+} concentrations: it collapses the transmembrane potential that leads to the release of cyctochrome c and activation of the apoptotic cascade (Szabadkai and Rizzuto, 2004).

In addition to ER and mitochondria the nuclear envelope, Golgi and lysosome related organelles have been implicated in maintenance of cellular Ca^{2+} homeostasis (Michelangeli, et al. 2005). Invaginations of the nuclear envelope of certain cell types contain InsP3Rs, RYRs and SERCA pumps and lumenal space that can act as a Ca2+ store (Gerasimenko, et al. 2003; Humber, et al. 1996; Xu, et al. 2001). The Golgi compartment also contains InsP3Rs (Surra and Wolff, 2000) and two types of Ca^{2+} pumps, the 'classical' SERCA type and the secretory pathway Ca^{2+} ATPase (SPCA), that is exclusively expressed in the Golgi (Wootton, et al. 2004). Also the lysosome and late endosomes have Ca^{2+} regulatory functions. They contain nicotinic acid dinucleotide phosphate

(NAADP) operated calcium channels and H^+–Ca^{2+} exchangers (HCE). A TRPC related Ca^{2+} channel mucolipin-1 has also been identified on lysosomes and late endosomes, and has been implicated in lysosome biogenesis (Piper and Luzio, 2004).

2.4 Cholesterol metabolism in the developing brain

Cholesterol is a key component of cellular membranes and regulates a variety of cellular functions. Most mammalian cells are able to synthesize cholesterol and also receive it from external sources. This process is highly regulated through feedback control in order to maintain the correct sterol balance prerequisite to proper cellular function. Cells synthesize cholesterol from acetyl CoA through a series of over 20 enzymatic reactions. 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) synthase and HMG CoA reductase are rate-limiting enzymes of the biosynthetic pathway and their activity is sharply reduced in response to external cholesterol input via the low-density lipoprotein (LDL) pathway. Once cholesterol is depleted from the extracellular milieu large numbers of LDL receptors are inserted on the plasma membrane. (Sato and Takano, 1995). External cholesterol is obtained from plasma via the classical route in which LDL particles, that are internalized in clathrin-coated pits via the LDL receptor mediated pathway, are hydrolyzed to free cholesterol in the lysosomes. Also alternative routes of uptake may be employed in which free cholesterol or cholesterol esters from lipoproteins are internalized on the cell surface, without endocytic uptake of the particle. (Azhar, et al. 1999). In the late endocytic organelles, free cholesterol is mobilized to other cellular membranes and this process requires the Niemann-Pick type C1 (NPC-1) protein. This membrane protein of the late endosomal/lysosomal membrane, defective in the Niemann-Pick disease type C, has been suggested to function as a regulator of cholesterol efflux from late endocytic organelles. However, the exact molecular function of the NPC-1 protein is still unclear (Kuwabara and Labouesse, 2002; Patterson, 2003).

The brain is enriched in cholesterol, the cholesterol concentration being up to ten times higher, compared to other organs (Dietschy and Turley, 2004). Cholesterol metabolism in the brain is segregated from other tissues because the CNS is isolated from the plasma by the blood–brain barrier that restricts the movement of plasma lipoproteins into the brain (Turley, et al. 1998). Therefore essentially all cholesterol is

synthesized within the brain. In the brain cholesterol is abundant in oligodendrocytes since the myelin sheath that they from contains 70–80% of total cholesterol (Dietschy and Turley, 2004). During brain development, oligodendrocytes wrap their plasma membrane around axons to produce the myelin sheath. It is a specialized membrane structure highly enriched in galactosylceramide (GalC) and cholesterol and arranged by the main proteinaceous component the myelin basic protein (MBP). It is the neuronal cells that induce the lipid condensation of the oligodendroglial membrane, during myelin formation. MBP is a essential part of this plasma membrane rearrangement, since the clustering is not observed in shiverer mice that lack MBP (Fitzner, et al. 2006). The rate of cholesterol synthesis in the CNS is highest in the developing brain, during the active myelination phase. This synthesis decreases upon maturation but cholesterol synthesis continues in the adult brain since also other cell types in the CNS, the astrocytes and neurons, require cholesterol for proper cellular function (Dietschy and Turley, 2004; Spady and Dietschy, 1983). The brain has an independent lipoprotein transport system in which glial cells produce ApoE (apolipoprotein E)-containing lipoproteins. ApoE is a component of lipoproteins that also contain cholesterol and phospholipids (LaDu, et al. 2000; Pitas, et al. 1987). The glia derived ApoE associated lipoprotein particles convey cholesterol to neurons for axonal growth and repair functions. Defects in cholesterol homeostasis in the brain have been associated with development of several neurological disorders, including Alzheimer's disease and NPC (reviewed in Vance, et al. 2005). Even though ApoE-containing lipoproteins produced by glial cells are normal in NPC, due to the cholesterol efflux regulation defect, cholesterol accumulates in somatic areas of neurons and the cholesterol content of axons is in contrast reduced. This results in defects in neuronal function, possibly in the recycling of synaptic vesicles (Vance, et al. 2006). ApoE-binding receptors participate in cholesterol uptake, and furthermore these receptors are required for signalling pathways in neurons during development (Trommsdorff, et al. 1999).

2.5 Cell death mechanims in neurons

Death of neurons occurs as a normal physiological process during development and as a pathological process in disease and injury. Neuronal death occurs by at least three different mechanisms: apoptosis, autophagy and necrosis. According to the current understanding these mechanisms share at least some overlapping regulators and fea-

tures. Apoptosis is central in neuronal death during development. It is characterized by morphological features such as DNA fragmentation, cytoskeletal collapse and membrane blebbing and also by activation of cellular pathways including the caspase (family of cysteine proteases) cascade, aided by adaptor proteins and mitochondria linked pathways mediated by the Bcl-2 family proteins (Yuan and Yanker, 2000; Chang and Reynolds, 2006). The second mechanism of cell death, autophagy, is also characterized by similar morphological features as apoptosis and in addition by the appearance of lysosome originated autophagic vacuoles and intracellular organelle swelling. The molecular mechanims regulating autophagy in neurons are still poorly understood but potential contributors include the PI3K pathway and the caspases (Guillon-Munos and van Bemmelen, 2005). Autophagy is a central housekeeping mechanism regulating turnover of intracellular organelles and some long-lived proteins but has also been implicated in neurodegenerative diseases (He and Klionsky 2006). Necrotic cell death is characterized by swelling and disruption of membrane integrity and distinguished from the other two types by the lack of lysosome as well as caspase involvement (Yuan, et al. 2003).

3. Cell biology of lysosomal storage disorders

3.1 Lysosomal storage disorders

Studying disease and pathophysiological mechanisms behind them provides information on disease mechanisms and in many cases normal cell physiology. Good examples of this principle are the lysosomal storage disorders (LSDs). Lysosomes are cellular organelles that are responsible for the degradation of various cellular metabolites. They are composed of a limiting external membrane containing intra-luminal vesicles. The lysosomes are estimated to contain 50-60 soluble acidic hydrolases (Journet, et al. 2002), 215 integral membrane proteins and 55 membrane associated proteins (Bagshaw, et al. 2005). Dysfunction in any of these components may cause lysosomal dysfunction leading to accumulation of undegraded metabolites and ultimately disease. Lysosomal storage disorders are often characterized according to the accumulating material or the defective protein. These comprise the sphingolipidoses, mucopolysaccharidoses, oligosaccharidoses and glycoproteinoses, lipidoses, diseases where the defective protein is an integral membrane protein and others. Over 40 lyso-

somal storage disorders (LSDs) that involve soluble hydrolases are known (Futerman and van Meer, 2004). The first LSDs were described already in the late nineteenth century; however, typically the molecular basis of the LSDs remained unclear until arrival of modern techniques. Even now when the genetic, molecular and biochemical background of many LSDs are understood, little is known about how accumulation of specific material to the lysosomes causes disease at the cellular level (Futerman and van Meer, 2004). Furthermore, questions remain open on the exact nature of the defect when accumulating material does not have an apparent relation to the defective enzyme as is the case with the topic of this thesis, the NCL group diseases.

3.2 Lysosomal storage and cellular function

How do cells maintain or lose their function in response to lysosomal storage? Several different strategies have been suggested ranging from general cellular responses to activation of disease specific mechanisms. Individuals affected by LSDs have in most cases lysosomal accumulation in all cell types. While the same defect is present in all cells, not all cell types become affected. Most LSDs have pronounced CNS manifestation but peripheral cells remain relatively unaffected. Examples include sphingolipidoses, lipidoses, glucoproteinoses and the NCL disorders (Futerman and van Meer, 2004; Mole, et al. 2005). Neurons seem particularly vulnerable to lysosomal storage but the exact reason for this is still unclear. The vulnerability may partly be caused by limited cell regeneration potential in the CNS and also by lack of compensatory cellular metabolic pathways (Futerman and van Meer, 2004; Tardy, et al. 2004). For most LSDs, the pathology mainly involves neuronal dysfunction rather than loss. The main exceptions to this are Niemann–Pick disease type C (NPC), in which differential loss of Purkinje neurons takes place, and the neuronal ceroid lipofuscinoses (NCLs) that are characterized by a massive neuronal loss at the cortex (Santavuori, et al. 1973; Walkley and Suzuki, 2004).

It has been suggested that accumulation of undegraded substrates in lysosomes leads to a intra-lysosomal protein response similar to which is seen in the ER in response to accumulation of unfolded proteins where several cellular pathways are activated leading to transcriptional changes and upregulation of degradative systems (see Zhang and Kaufman, 2006 for review). It is also possible that the aggregations in the lyso-

Disease Category by Defect	Disease	Deficiency	Main storage material	Disease Category by Storage Type
Primary lysosomal hydrolase defect	Gaucher disease	Glucosylceramidase	Glucosylceramide	**Sphingolipidose**
		Saposin-C activator	Glucosylceramide	‘’
	GM1 gangliosidosis	β–Galactosidase	GM1 ganglioside	‘’
	Tay-Sachs disease	β–Hexosaminidase A	GM2 ganglioside/glycolipid	‘’
	Sandhoff disease	β–Hexosaminidase A&B	GM2 ganglioside/glycolipid	‘’
	Fabry disease	α–Galactosidase A	Globotriasylceramide	‘’
	Nieman-Pick A & B	Sphingomyelinase	Sphingomyelin	‘’
Trafficking defect for lysosomal enzymes	Mucolipidosis II&IIIA	*N*-acetyl glucosamine phosphoryl transferase	Lipid/mucopolysaccharides	
Defect in lysosomal enzyme protection	Galactosialidosis	Protective protein cathepsin A (β–Galactosidase & neuraminidase)	Lipid/mucopolysaccharides	**Mucopolysaccharidose**
Defect in soluble non-enzymatic lysosomal proteins	GM2 activator protein deficiency	GM2 activator protein	GM2 ganglioside/glycolipid	**Sphingolipidose**
	Sphingolipid activator protein deficiency	Sphingolipid activator protein	Glycolipids	‘’
	Late infantile NCL	CLN5	Subunit c of mitochondrial ATP syntahse	
Transmembrane protein	Nieman-Pick C	NPC-1, NPC-2	Cholesterol and sphingolipid	
	Juvenile NCL	CLN3	Subunit c of mitochondrial ATP syntahse	
	Salla disease	Sialin	Sialic acid	
Others	Mucosulphatidosis	Multiple sulpha-tases	Sulphatides	
	Infantile NCL	PPT1	Sphingolipid activator proteins A and D	
	Pompe disease	α–Glucosidase	Glycogen	**Oligosaccharidose & Glycoproteinose**

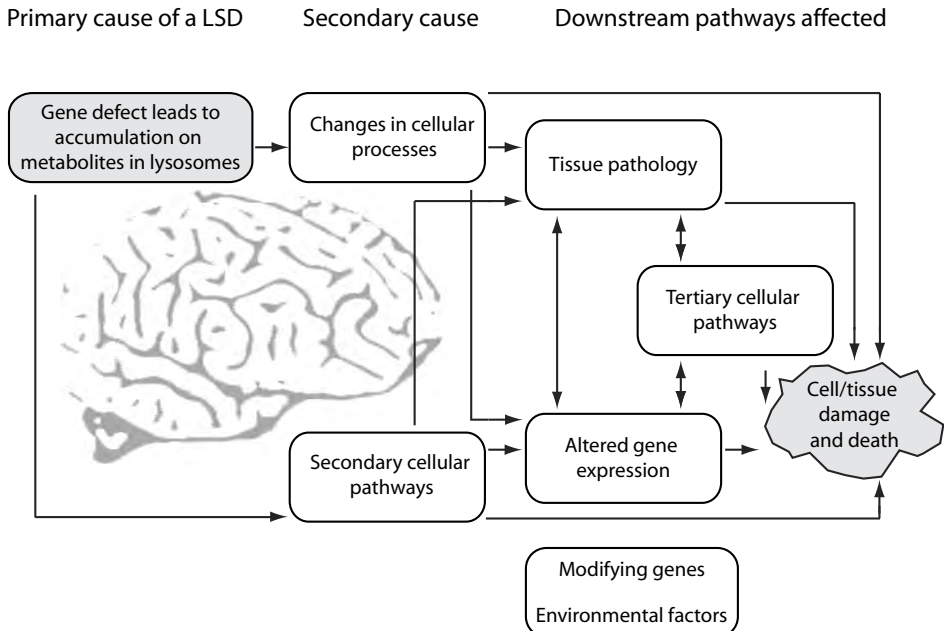
Table 1. Lysosomal Storage disorders classified according to molecular defect and storage material

Adapted from: Jeyakumar et al. 2005; Futerman and van Meer 2004

somes themselves are not pathogenic to the cells but rather diffuse mutant proteins in the cytosol (Arrasate, et al. 2004; Bjorkoy, et al. 2005; Tanaka, et al. 2004; Taylor, et al. 2003). In this case autophagic systems would be activated. Autophagy is a rather non-selective process in which cellular components are dergaded in the lysosomes. In the autophagy event small parts of the cytoplasm are sequestered into autophagosome vesicles which then fuse with the lysosomes and the contents is degraded (Klionsky, 2005; Levine and Klionsky, 2004). Autophagy mediated protein quality control is especially important in neural cells and impairment of autophagy can contribute to and even worsen the accumulation in neurodegenerative diseases (Fortun, et al. 2003; Ravikumar, et al. 2002). Activation of autophagy might facilitate the removal of intracellular protein aggregates (Jellinger and Stadelmann, 2000; Larsen and Sulzer, 2002). Autophagy has been shown to be involved in the abnormal storage accumulation in lysosomal storage disorders, including NCL (Cao, et al. 2006; Koike, et al. 2005; Tardy, et al. 2004).

Even when unmetabolized substrates remain the primary cause of lysosomal storage disease and while general responses to accumulations in the lysosomes or cytosolic misfolded proteins may play a role in disease pathology, the wide range of disease symptoms in LSDs indicates that a plethora of secondary and even tertiary cellular and biochemical pathways are also activated. Moreover, many LSDs are characterized by phenotypic variability: phenotype cannot be predicted on the basis of the gene defect or even accumulating substance. Patients with similar genetic background show variable clinical symptoms presumably due to modifying genes and environmental factors (Futerman and van Meer, 2004). The possible pathways from primary defect to pathology are presented in figure 5.

Figure 5. Pathways to Pathology in Lysosomal Storage Diseases.



3.3 Mechanims of lysosomal pathology

The pathogenic cascades resulting from lysosomal storage in many LSDs have been shown to share pathological features with common neurodegenerative diseases. These include altered calcium homeostasis, altered endosomal/lysosomal function and inflammation in the CNS. (Jeyakumar, et al. 2005). Altered calcium homeostasis has

been described in several LSDs, including the glycosphingolipid (GSL) storage disorder Gaucher's disease (Korkotian, et al. 1999; Lloyd-Evans, et al. 2003) and the ganglioside storage disorder Sandhoff's disease (Pelled, et al. 2003). In the Gaucher mice cells, an ER calcium channel agonist, the ryanodine reseptor, induced enhanced calcium release from the ER internal stores. In addition, increased sensitivity to glutamate-mediated neurotoxicity, was present. A calcium-related phenotype was identified also in the mouse model of Sandhoff disease. In this model a profound reduction in the rate of calcium uptake but no difference in the rate of calcium release from the ER, was observed. This defect was attributed to modulation of the sarcoendoplasmic reticulum calcium ATPase (SERCA) by the storage ganglioside GM2.

Altered endosomal/lysosomal function contributes to pathology in LSDs and defects have been demonstrated in various steps of endosomal trafficking. Altered trafficking of sphingolipids, connected to either elevated cholesterol levels or altered cholesterol distribution occurs in several storage disorders (Chen, et al. 1999; reviewed in Olkkonen and Ikonen, 2006). Defective trafficking due to failure in correct production or recognition of endocytic sorting signal is present in some LSDs: examples include Mucolipidosis II (I-cell disease) and related milder disorders. I-cell disease is cause by impaired activity of the N-acetylglucosamine-1-phosphotransferase enzyme. This results in missorting of multiple lysosomal hydrolases, causing leakage of these proteins from cells and deposits of undegraded material (Glickman and Kornfeld, 1993; Varki, et al. 1981). This enzyme catalyses the addition of mannose 6-phosphate (M6P) on lysosomal proteins, that are recognized in the *trans*-Golgi network (TGN) or at the cell surface by M6P receptors (MPRs) and routed to lysosomes. Additionally, defects in mucolipidosis II and IIIA were recently pinpointed to the gene encoding the α and ß subunits of this enzyme (Kudo, et al. 2006; Raas-Rothschild, et al. 2004; Tiede, et al. 2005). Trafficking defects, characterized as delay in several endocytic pathways, have been described in NCL group of diseases (Heine, et al. 2004; Luiro, et al. 2004). Trafficking defects have also been suggested to contribute specifically to the neuropathology of LSDs. For example in normal neurons, the late endosomal/lysosomal compartment is primarily confined to the soma, however in cultured neurons lysosomes have also been reported in the axon (Overly and Hollenbeck, 1996). The lysosomes in the axon can potentially fuse with endosomes derived from synaptic areas, and it has been suggested that some degradative events might occur already while these

organelles travel to the cell body. The lysosomal storage in LSDs might cause defects in the retrograde transport of lysosomes, possibly by preventing the acidification of endosomes derived from the axon terminal. Alternatively retrograde transport of components along the axon might be impaired by storage in the cell body. Abnormal synaptic function has been shown in gangliosidoses and implied in NPC (Karabelas and Walkley, 1985; Karten, et al. 2006; Walkley and Suzuki, 2004).

Abnormal inflammatory responses in the brain are common in LSDs. They are present in glycosphingolipidoses, the gangliosidosis Sandhoff disease and in all types of NCLs (Cooper, et al. 2006; Jeyakumar, et al. 2005). Astrocytosis and microglial activation are found in several NCLs in patient autopsy material as well as mouse models (Cooper, et al. 2006; Mitchison, et al. 2004; Pontikis, et al. 2004).

4. Infantile neuronal ceroid lipofuscinosis

4.1 Neuronal ceroid lipofuscinoses

The neuronal ceroid lipofuscinoses (NCLs) are a group of autosomal, recessively inherited, neurodegenerative diseases with an onset usually in the childhood. At least ten different NCL diseases classified according to the gene defect, varying in severity, have been described. To date approximately 160 NCL causing mutations have been found in seven human genes (*CLN1, CLN2, CLN3, CLN5, CLN6, CLN7, CLN8 and CLN10*, http://www.ucl.ac.uk/ncl) of which *CLN7* and *CLN10/CTSD* are the newest (A. Lehesjoki et al., unpublished; Siintola, et al. 2006; Steinfeld, et al. 2006). At least two more NCL genes, *CLN4* and *CLN9*, are anticipated to exist. In Table 2 are presented the human NCL genes, associated clinical phenotype and storage material. Despite being a genetically heterogeneous group, the NCLs share morphological as well as histopathological and clinical characteristics. Two most essential findings in all NCLs are loss of neurons in the central nervous system mainly in the cerebral and cerebellar cortices and accumulation of autofluorescent material, so called ceroid-lipopigments, in most tissues (Goebel, et al. 1999). Clinically, the NCLs are marked by several symptoms caused by the neurodegeneration commencing with visual impairment leading to blindness, sleep problems, motor abnormalities, epilepsia, dementia and eventually premature death (Santavuori, et al. 2000). While the disease phenotype

is quite constant across the childhood NCLs, precluding *CLN8* or Northern epilepsy, the age of onset and course of the disease varies. The NCLs were originally classified based on the clinical onset of symptoms to four main forms: infantile (INCL), late-infantile (LINCL), juvenile (JNCL) and adult forms (ANCL;CLN4, gene not identified). In recent years several so-called variant forms have been recognised having later onset than the classical forms or being less severe or protracted in course (Goebel, et al. 1999; Mole, et al. 2005). The NCLs occur worldwide and are considered most common progressive brain disorders in children (Haltia, 2003; Mole, et al. 2005). They are especially common in Finland, the most common types being INCL (incidence 1:20 000) and JNCL (incidence 1:21 000). The classic late infantile form NCL (LINCL) is rare, whereas the Finnish variant LINCL ($vLINCL_{Fin}$) is more common (Santavuori, 1988).

Table 2. The neuronal ceroid lipofuscinoses

Gene	Protein	Type	Main storage protein	Ultrastructure	Clinical phenotype
CLN1	**PPT1**, palmitoyl protein thioesterase 1	Soluble lysosomal & neuronal extensions	Saposin A&D	GROD	**INCL**, also LINCL and ANCL
CLN2	**TPP1**, tripeptidyl peptidase	Soluble lysosomal	SCMAS	CL	**LINCL,** also JNCL
CLN3	**CLN3**	Transmembrane	SCMAS	FP (CL, RL)	**JNCL**
CLN4	not known			FP, granular	**ANCL**
CLN5	**CLN5**	Soluble lysosomal/ transmembrane	SCMAS & Saposin A&D	RL, CL, FP	**LINCL,** also Finnish variant and JNCL
CLN6	**CLN6**	Transmembrane, ER resident	SCMAS	RL, CL, FP	**LINCL**, also EJNCL Indian/Costa Rican/ Czech Gypsy variant, JNCL
CLN7	unpublished	unpublished	SCMAS	RL, CL, FP	**LINCL**, Turkish variant
CLN8	**CLN8**	Transmembrane, ER resident	SCMAS	CL-like granular	**Northern epilepsy,** also Turkish variant, JNCL
CLN9	unpublished				
CLN10	**CTSD** cathepsin D	Soluble lysosomal	Saposin A&D	GROD, FP	**congenital NCL**

Classification of the neuronal ceroid lipofuscinoses. Genes and respective proteins, main storage protein, ultrastructural phenotypes of storage bodies and clinical phenotypes are presented.
Saposin, sphingolipid activator protein; SCMAS, subunit c of mitochondrial ATP synthase; GROD, granular osmiophilic deposits; CL, curvilinear profiles; FP, fingerprint bodies; RL, rectilinear complexes

(Vesa et al. 1995; Sharp et al. 1997; Sleat et al. 1997; Järvelä et al. 1998; International Batten Disease Consortium. 1995; Berkovic et al. 1988; Savukoski et al. 1998; Wheeler et al.1999; Ranta et al. 1997; Schulz et al. 2006; Siintola et al.2006)

NCLs are considered as lysosomal diseases and have some properties that group them with the classical lysosomal storage diseases (LSDs), however also many distinctive characteristics are observed. In NCLs the ceroid-lipopigments are accumulated in the lysosomes. The diseases have a spectrum of severity being present in the infantile, juvenile and adult forms, the most severe infantile form having CNS symptoms and

whereas in the adult form peripheral symptoms predominate. The NCL disorders are recessively inherited and many of the NCL proteins are present in the lysosomes. These are characteristics of many LSDs (Futerman and van Meer, 2004). The NCLs cannot, however, be treated as classical LSDs, such as sphingolipidoses and mucopolysaccharidoses, where enzyme or transporter deficiency/dysfunction leads to accumulation of the specific undergraded metabolites in lysosomes. The accumulating material in NCLs is not a disease specific substrate. The main accumulating material in all NCLs except INCL and CONCL is the subunit c of mitochondrial ATP synthase which is present in other LSDs as well as the main storage proteins sphingolipid activator proteins A and D in INCL and CONCL. Also the intracellular localization of at least two NCL proteins is not in the lysosomes but neuronal extensions.

4.2 Clinical and neuropathological findings in INCL patients

Infantile neuronal ceroid lipofuscinosis (INCL) (MIM 256730) is caused by mutations in the *CLN1* gene, residing on chromosome 1p32, encoding Palmitoyl protein thioesterase 1 (PPT1, EC 3.1.2.22) (Järvelä, et al. 1991; Vesa, et al. 1995). INCL is the most severe of NCL types. The clinical spectrum is wide depending on the mutation of the *CLN1* gene, ranging from infancy to adulthood.

The Infantile onset form predominates in Finland. INCL children develop quite normally for the first year of life but development starts to slow down during the second year. First symptoms start at the age of 10-18 months and are followed by slowing down of development, visual failure and epileptic seizures (Santavuori, et al. 2000). Symptoms such as restlessness and sleep problems are thought to be of thalamic origin since magnetic resonance imaging (MRI) and electroencephalography (EEG) findings indicate thalamic dysfunction (Vanhanen, et al. 1994; Vanhanen, et al. 1995). Retinas show progressive deterioration as seen by electroretinography (ERG) findings (Vanhanen, et al. 1997). Loss of neurons is observed in most parts of the brain, however the extent of neuronal loss varies. The cerebral cortex is almost totally lost and the cerebellum is also atrophied whereas the brain stem and spinal cord appear almost normal. Consistently with the observed visual impairment the optic nerves are severely atrophic and the myelin sheaths are replaced by glial tissues (Tarkkanen, et al. 1977). Clustering of glial cells filled with the storage material is present in all brain

areas (Haltia, et al. 1973). EEG inactivity, indicating total loss of cortical function, is also seen from three years onwards (Santavuori, et al. 1973). Patients have low GABA concentrations in their cerebrospinal fluid (CSF) (Santavuori, et al. 1993). Premature death follows not until ages between 9 and 11 years, mainly due to neurodegeneration being present mainly in the cerebral and cerebellar cortices leaving the brain stem almost intact.

4.3 Intracellular ceroid-lipofuscin storage in INCL

Most cell types of INCL patients accumulate membrane bound storage deposits, called granular osmiophilic deposits (GRODs). These deposits are disease specific. They are always present in a disease caused by mutations in the *CLN1* gene and have a similar ultrastructure independent from the clinical subtype (Van Diggelen, et al. 2001). The deposits reside in the lysosomes and resemble autofluorescent lipofuscin pigments that accumulate in cells during normal aging. However, GRODs can be differentiated from normal aging pigment by virtue of lipid droplets they contain (Lu, et al. 1996; Tyynelä, et al. 1993). GRODs have a packed globular structure which is more granular and tightly packed in neurons and coarser and looser in non-neuronal cells. GRODs can be detected for diagnostic purposes in blood lymphocytes and skin dermal cell types (Mole, et al. 2005). Granular lipopigments appear in mural cells of chorionic vessels after the 8th week of gestation, but the time of appearance in non-chorionic fetal tissue is currently unknown (Wisniewski, et al. 1998).

Major part of the storage material comprises of two sphingolipid activator proteins saposins A and D. Saposins are small glycoproteins that promote the degradation of sphingolipids (Fürst, et al. 1988). The normal lysosomal function of saposins is to activate lysosomal hydrolases involved in glycosphingolipid degradation. The saposins are extremely hydrophobic and associate with the lysosomal membrane. Due to this hydrophobicity the saposins have a tendency to self-aggregate. In most lysosomal storage diseases the levels of saposins are near normal (Morimoto, et al. 1990). However, accumulation of various saposin species has been shown in some lysosomal storage diseases namely Tay Sachs, Sandhoff, Niemann-Pick and Gaucher disease. Typical for INCL is the specific massive accumulation of saposins A and D but not the other saposin species (Tyynelä, et al. 1993). INCL cells also show some lipid

accumulation leading to dramatic changes in the phospholipid molecular species of INCL brains (Käkelä, et al. 2003). In contrast to the other NCL disorders as well as several other lysosomal storage disorders, INCL cells do not accumulate the subunit c of the mitochondrial ATP synthase (Tyynelä, et al. 1993). The reason for saposin accumulation in INCL is still unclear. However, abnormal saposin processing connected to defects in endocytosis, presented in this thesis work, may be the reason underlying the accumulation (Ahtiainen, et al. 2006). Further studies are still needed to unravel the mechanisms of neuronal death present in INCL, to pinpoint the physiological role of the PPT1 enzyme and to clarify if storage is the reason or just the end result in the cascade leading to cell death.

4.4 Saposins, biosynthesis and pathway to lysosomes

Sphingolipids and glycosphingolipids are components of the cellular membranes. Membrane lipid content is dynamically regulated and sphingolipids are constitutively degraded in a process that takes place in intra-endosomal and -lysosomal compartments. Degradation requires water-soluble acid exohydrolases, anionic phospholipids and sphingolipid activator proteins such as the SAPs and GM2 activator proteins. Sphingolipid activator proteins are proteins that have the ability to perturb the membrane and bind lipids. They have specificities for different lipids and activating enzyme that catalyses the degradation (Kolter and Sandhoff, 2005). The four mature saposin species A, B C and D are generated by cleavage from a common precursor prosaposin (proSAP) (Fürst and Sandhoff, 1992; Sandhoff and Kolter, 1997). The proSAP 65 kDa precursor has two alternative trafficking routes: It is transported directly from the trans-Golgi network (TGN) to the lysosomes via a mannose 6-phosphate (M6P) dependent pathway. The precursor can also traffic to lysosomes independently of M6P, in which case the precursor is modified into a 73 kDa form and secreted. The secreted form is endocytosed back into the cell by receptor mediated endocytosis utilizing the LDL receptor related protein (LRP) or M6P receptors and then transported via the endocytic pathway to the lysosomes. In the endo-lysosomal compartments proSAP is proteolytically cleaved to four small polypeptides of 8–11 kDa, known as saposins A–D (Hiesberger, et al. 1998). The details of the cleavage process including the exact order of events and the compartment, in which it takes place, are still poorly characterized (Vielhaber, et al. 1996).

4.5 PPT1 protein

4.5.1 Structure and function of PPT1

PPT1 is a depalmitoylating enzyme, which removes palmitate groups from S-acylated substrates *in vitro* (Camp, et al. 1994). However, the specific *in vivo* substrate of the enzyme is unknown. The PPT1 polypeptide consists of 306 amino acids and has a signal sequence of 25 aa in the N-terminus that translocates the protein into the ER lumen. PPT1 has three glycosylation sites and two sequence motifs that are characteristic of thioesterases (Schriner, et al. 1996). The crystal structure of the mature bovine PPT1 has been determined. It is 95% identical to the human PPT1. It is a monomeric globular protein and structure has a α/β-hydrolase fold with the active site, a conserved catalytic triad, composed of Ser115-Asp233-His289. Proper glycosylation is required for stability and activity of the enzyme (Bellizzi, et al. 2000).

The exact physiological function and substrates of PPT1 have remained elusive. PPT1 was originally characterized as a classical soluble lysosomal enzyme. In non-neuronal cells it is guided to the lysosome via the M6P receptor pathway as a typical lysosomal hydrolase (Hellsten, et al. 1996; Verkruyse and Hofmann, 1996). PPT1 plays a role in the degradation of lipid-modified proteins, cleaving fatty acids (usually palmitate) from cysteine residues (Lu, et al. 1996). Eligible *in vitro* targets for PPT1 comprise proteins as well as peptides including the proto-oncoprotein H-Ras, palmitoyl-CoA and *S*-palmitoyl thioglucoside (Camp, et al. 1994; van Diggelen, et al. 1999). PPT1 has also been shown to depalmitate several neuron-specific peptides, for example, GAP-43 (growth-associated protein) and rhodopsin and reduce their membrane association (Cho, et al. 2000a; Cho, et al. 2000b).

4.5.2 PPT1 expression and intracellular localization

PPT1 is an abundant protein expressed in most tissues types. The highest expression is seen in spleen, brain, lung and testis (Camp, et al. 1994; Suopanki, et al. 1999). On whole tissue level in mouse and rat brains PPT1 expression varies depending on the developmental context. The expression increases during the maturation of the central nervous system reaching a peak level in early adulthood. PPT1 expression parallels

synaptogenesis events both spatially and temporally (Isosomppi, et al. 1999; Suopanki, et al. 1999). On the cellular level PPT1 has a differential intracellular distribution depending on the cell type. In non-neuronal cells PPT1 resides in the endolysosomal compartment and uses the classical M6P receptor-mediated pathway to reach the lysosomes (Hellsten, et al. 1996; Verkruyse and Hofmann, 1996). In contrast, in neurons PPT1 is present in presynaptic regions of axons. More specifically it is enriched in synaptosomes and is associated also with the synaptic vesicles (Heinonen, et al. 2000; Lehtovirta, et al. 2001; Ahtiainen et al. 2003) as shown by cell fractionation and cryoimmunoelectron microscopy studies. A similar differential distribution in neuronal and non-neuronal cells has also been reported for other proteins associated with neurodegenerative diseases such as presenilin (Kamal, et al. 2001) and another NCL protein CLN3 (Luiro, et al. 2001).

5. Unravelling the INCL Disease Mechanism

The exact disease mechanism in INCL and the significance of lysosomal storage to disease pathology as well as reason for accumulation of the specific components in lysosomal storage in INCL is still unclear. Also the exact intracellular role of the PPT1 protein and its significance in the neuronal physiological function are only beginning to be understood. A highly specific pattern of neuronal loss is present in the central nervous system and specific alterations in brain metabolites characterize the INCL disease. Extensive studies have been conducted both of patient tissues and various cell models as well as transgenic mouse models to characterize the sequence of cell loss and to understand the underlying molecular mechanisms in INCL pathogenesis.

When studying a rare neurodegenerative disease a major obstacle is getting the material for the research. Even though non-invasive advanced functional imaging techniques are emerging, studying cellular pathogenetic mechanisms and function at the molecular level necessitates the use of live cells. Non-neuronal patient cells, such as the skin fibroblast cells, can be utilized. They are relatively easy and non-invasive to obtain, can be readily grown in culture and are amenable to various cell biological approaches. However, when studying a neurodegenerative disease the underlying defect can have very different manifestations in different cell types. Therefore it is also crucial to study the affected cells, in the case of INCL the neurons. Since central nerv-

ous system neurons are relatively inaccessible, a genetically engineered experimental mouse disease model is an invaluable tool to study early pathogenic events and also universal mechanism of neuronal death and function. Once landmarks for disease progression are established also therapeutic approaches and their efficacy can be tested.

5.1 Studies in patient tissues and cell models

Based on patient studies several mechanisms have been suggested to contribute to pathogenesis and ultimately death of neurons in INCL. Connections to energy metabolism and calcium regulation are derived from studies by Das and colleagues (Das, et al. 1999) in INCL patient fibroblasts. They have shown that basal ATP synthase activity is reduced and regulation of the enzyme is absent in INCL cells. Activities of respiratory chain complexes II and IV were also reduced. Furthermore ATP synthase was down-regulated under conditions of anoxia and was up-regulated in response to calcium. Several studies have shown that the INCL brain has alterations in the balance of various metabolites: INCL patient brains have decreased levels of *N*-acetylaspartate (NAA) and respectively proportional increase in myoinositol and lipids, decrease in γ-aminobutyric acid (GABA), glutamine and glutamate (Sitter et al. 2004). Patient brains also contain proportionally more phosphatidylcholine (PC), and less phosphatidylethanolamine (PE) and phosphatidylserine (PS). The INCL cortex loses up to 65% of the normal phospholipid content, and the proportions of polyunsaturated molecular species decrease dramatically. Furthermore, Lysobisphosphatidic acid (LBPA, major component of the late endosomal membrane) is highly elevated in the INCL brain (Käkelä, et al. 2003). It is highly probable that these changes alter signaling and membrane trafficking in neurons contributing to disease pathology.

Tissue level studies on patients have revealed distinct patterns of neuronal degeneration. In the well studied hippocampus, the CA2-CA4 regions show degeneration whereas the CA1 region is relatively spared, characteristic specifically to NCLs. Typical patterns of astrocytosis and activated microglia are also present representing abnormal inflammatory responses. Inhibitory interneurons show graded severity of loss with the relative sparing of calretinin-positive interneurons in all regions. The selective pattern of degeneration in interneuron populations has been suggested to associate with differential buffering capacities of cytosolic calcium-binding proteins.

(Cooper, 2003; D'Orlando, et al. 2002; Haltia, et al. 2001). Cell death mechanisms, neuronal apoptosis and the caspase cascade, have been studied in INCL patient autopsy brain samples. Apoptotic cell death in INCL brain has been linked with low levels of insulin like growth factor-1 shortage (Riikonen et al. 2000). Neuronal apoptosis in INCL brain has been shown to be caused by ER stress mediated caspase-4 activation that leads to apoptosis via caspase-3. Activated caspases 3 ja 4 were detected in the hippocampus and cortex as well as apoptosis markers poly-ADP-ribose polymerase (PARP) and X-box binding protein-1 (XBP1), a marker of ER stress induced unfolded protein response. These results were functionally confirmed in lymphoblasts and patient fibroblasts. Also abnormally high amounts of the palmitoylated protein GAP-43, possibly contributing to ER-stress, was detected in the brain (Kim, et al. 2006a). ER stress induced reactive oxygen species increase and calcium homeostasis impairment leading to caspase-9 activation have also been implicated in rapid neurodegeneration in INCL brain (Kim, et al. 2006b).

A range of cell level studies have been conducted in cell models. The role of PPT1 in the metabolism of lipid-modified proteins was originally shown in metabolic labeling studies in patient INCL fibroblast cells: Lipophilic compounds, migrating as small peptides, were shown to be derived from a process of lysosomal degradation of lipid-modified proteins (Lu, et al. 1996). The pH regulation has been studied in INCL patient cells and the lysosomal pH has been shown to be elevated (Holopainen, et al. 2001). The interactions and common pathways of the different NCL proteins have also been characterized in patient cells. A recent cross correction study on apoptosis and cell growth, showed that PPT1 and the *CLN2* protein TPP1, are to an extent interchangeable and they also were shown to co-immunoprecipitate (Persaud-Sawin, et al. 2007). PPT1 expression has been shown to protect from cell death, whereas antisense PPT1 treatment induced apoptotic cell death in LA-N-5 neuroblastoma cells (Cho, et al. 2000a; Cho, et al. 2000b).

5.2 Reproducing the disease, characterization of mouse models

To address the role of PPT1 in neurons as well as mechanisms leading to INCL disease pathology genetically engineered disease model mice lacking Ppt1, the murine

counterpart of the human PPT1, have been produced: the *Ppt1*$^{tm1Hof-/-}$ mouse by Gupta and colleagues (Gupta, et al. 2001) and the *Ppt1*^{Deltaex4} mouse by Jalanko and colleagues published in 2005 (Jalanko, et al. 2005) and functionally characterized in this thesis work. (Full descriptions of the NCL mouse models in the NCL Mouse Model Database http://www.ucl.ac.uk/ncl-models/). The mice have pathology similar to the human INCL disease. They accumulate autofluorescent GROD storage material, develop blindness, motor deficits and myoclonic seizures and die prematurely. Thorough neuropathological characterization of the mouse models has also brought understanding of several novel features in NCL pathology. These include eye-opening findings about the nature of neurodegeneration in INCL; the neuronal death is highly selective having a specific pattern and temporal course. Also early events of the pathogenesis are starting to be unveiled: the exact tissue pathology modelling has revealed the thalamic region central in early disease progression. (Bible, et al. 2004; Cooper, et al. 2006; Kielar, et al. 2007) Findings in patient brain autopsy samples, including the pattern of neuronal loss and abnormal inflammatory responses through early glial activation, are being recognized and further characterized in the mouse models.

Neurodegeneration has been characterized in the Ppt1-deficient mouse models with histological methods. The *Ppt1*^{Deltaex4} mouse shows severe reduction of brain weight and loss of GABAergic interneurons in several brain areas, most prominently in the cortex and cerebellum, at the age of six months (Jalanko, et al. 2005). The neuropathological features of the *Ppt1*$^{tm1Hof-/-}$ mouse have been more widely characterized and the loss of GABAergic interneurons is evident in this mouse model in the hippocampus and cerebellum at the age of 6 moths (Gupta, et al. 2001). Interneuron populations exhibit a graded vulnerability dependent on location, and which calcium binding protein or neuropeptide(s) is expressed by subsets of these neurons. The mice show severe loss of specific neuron populations: severe atrophy was observed in the primary visual and somatosensory barrel field with significant involvement of the lamina V pyramidal neurons and lamina IV granule neurons. The atrophy also involves subcortical areas especially the thalamic region (Bible, et al. 2004). Evidence in other NCL mouse models, namely the *CLN3* mouse (Pontikis, et al. 2004; Weimer, et al. 2006), suggests that changes in sensory cortex follow the loss of corresponding sensory relay neurons within the thalamus. Therefore it seems that cellular location is the major determinant of neuron survival (Cooper, et al. 2006). Synaptic involvement in early

in disease pathogenesis has also been implicated. Cultured neurons of the *Ppt1*$^{tm1Hof-/-}$ knock-out mice have a reduced synaptic vesicle pool size and the colocalization of Ppt1 with presynaptic markers is lost by expression of mutant proteins (Lehtovirta, et al. 2001; Virmani, et al. 2005).

The neuronal loss is in parallel with widespread astrocytic but more restricted microglial activation. Upregulation of GFAP, an astrocyte marker, expression has also been shown in microarray studies of Ppt1-deficient mouse models (Elshatory, et al. 2003). Studies on the mechanisms of neuronal death in the *Ppt1*$^{tm1Hof-/-}$ knock-out mice have revealed morphological changes that have been correlated with ER stress induced unfolded protein response similarly to INCL patient brain autopsy samples. ER stress induced apoptosis is mediated via the caspase cascade involving caspases 3 and 12 in mice (Zhang and Kaufman, 2006).

The mouse disease has recently been modeled in the context of efficiently charactering the outcome of treatment strategies. The gene therapy studies with an adeno-associated virus encoding Ppt1 (AAV2-Ppt1), have shown improved histological and behavioral parameters as well as decreased neurodegeneration through protective effects. Unfortunately, no significant improvement in the seizure phenotype or increased longevity could be shown. There are implications that the diffusion/trafficking properties of Ppt1 differ from other lysosomal enzymes. Therefore, these studies have emphasized the need for higher levels or a broader distribution of Ppt1 expression for a more complete correction of the disease. (Griffey, et al. 2004; Griffey, et al. 2006)

Aims of the present study

This study was carried out to characterize the role of PPT1 in early neurodegenerative events in INCL. The impact of specific protein storage to pathogenesis and the relationship to PPT1 were also addressed.

The specific aims of this study were the following:

- To determine the exact intracellular localization of PPT1 protein in neurons
- To analyse Ppt1 expression during neuronal maturation in primary neuronal cultures
- To study intracellular trafficking pathways in PPT1 deficient patient cells
- To examine the role of lysosomal storage proteins, the saposins, in INCL pathogenesis and the potential relationship between PPT1 and saposins
- To characterize functional properties of *Ppt1*^{Deltaex4} mouse neurons
- To clarify mechanisms of early pathogenesis in INCL utilizing *Ppt1*^{Deltaex4} mouse neurons

Materials and methods

1. Published materials and methods

Materials and methods used in this thesis are described in the original publications.

Table 3.

Material or Method	Original publication
Adenovirus -mediated gene expression	**I**
Assay for PPT1 activity	**I**
Fura-2 AM ratiometric calcium imaging	**III**
Cloning of cDNAs or other plasmid constructs	**I**
Confocal microscopy	**I, II, III**
Cholesterol biosynthesis in neuronal cultures	**III**
Dissection and culturing of primary retinal, cortical and hippocampal neurons	**I, II, III**
DNA extraction	**III**
Electron- and immunoelectron microscopy	**I**
Electrophysiological recordings by whole cell patch clamp method	**III**
Transcript profiling and data analysis	**III**
Immunofluorescence quantifications of confocal images	**II**
Immunoprecipitation	**II**
Mouse brain acute slice preparation	**I, II, III**
Metabolic labelling	**II**
Pharmacological agents affecting intracellular trafficking	**II**
Primary fibroblast cultures	**II**
Proliferation assay of neuronal stem cells	**III**
Protein detection by direct and indirect immunofluorescence	**I, II, III**
Protein detection by Western analysis	**II**
Receptor-mediated endocytosis assay	**II**
Non-receptor mediated endocytosis assay	**II**
RNA extraction	**III**
Semliki Forest virus –mediated gene expression	**I**
Transferrin endocytosis and recycling assay	**II**
Transient transfections	**II**

2. Ethical aspects

This study has been evaluated and approved by the Laboratory Animal Care and Use Committee of the National Public Health Institute, Helsinki. This study has been conducted following good practice in laboratory animal handling and according the regulations for handling genetically modified organism.

Results and discussion

1. Localization and expression of PPT1 in neuronal synapses (I)

Prior to this study the *CLN1* gene defective in INCL had been identified (Vesa, et al. 1995). Intracellular localization and transport of the PPT1 protein had been pinpointed in non-neuronal cells (Hellsten, et al. 1996; Verkruyse, et al. 1996) and studies on neuronal expression had been initiated. On tissue level PPT1 expression was shown to be developmentally regulated and coincide with synaptogenesis. (Isosomppi, et al. 1999; Suopanki, et al. 1999; Heinonen, et al. 2000). Main part of the INCL disease symptoms arise from the neuronal dysfunction and ultimately degeneration whereas non-neuronal cell types remain, apart from storage material accumulation, relatively unaffected. Because of the severe neuronal manifestation PPT1 is postulated to have a crucial role in the development and survival of neurons. Intracellular localization studies in neurons suggested presynaptic localization and accordingly it was anticipated that in neuronal cells PPT1 has extra-lysosomal functions, possibly associated with the development or maintenance of the synaptic machinery. Therefore, it was of main importance to elucidate the exact intracellular location and expression patterns of PPT1 in normal neurons. In this study, neurons from the retina, hippocampus, and cortex were used because these cell types are most affected and ultimately lost in the course of INCL disease. PPT1 activity was measured during neuronal maturation in culture. Quantitative measurements of polarized protein expression in axons versus dendrites were performed and the intracellular localization of endogenous and over expressed protein was studied.

1.1 Increase in PPT1 activity by neuronal maturation

To analyse the properties of PPT1, enzyme activity was monitored in primary mouse embryonic retinal and hippocampal neuron cultures at different maturation stages. Measurements were made utilizing an artificial substrate, 4-methylumbelliferyl-6-thiopalmitoyl-β-D-glucoside. This sensitive fluorogenic substrate is also used for pre- and postnatal enzyme analyses of INCL (van Diggelen, et al. 1999). Samples from immature and mature cultures were studied. The measurements showed an overall

higher level of PPT1 activity in retinal neurons than in hippocampal neurons. However, the differences between groups did not reach statistical significance ($P > 0.05$) (I, Fig. 1). Activity measurements were made from primary neuron cultures, which often show heterogeneity explaining the relatively high variance within each group. Furthermore, the higher level of expression of PPT1 in retinal neurons was verified by Western blot analysis (I, Fig. 2A).

A rise in PPT1 activity with time was observed in both cell types. Especially in retinal neurons, a pronounced increase in PPT1 activity was detected in mature cultures at 13 DIV. PPT1 preceded the expression of synaptophysin, a marker for mature synapses. The increase in activity of PPT1 also temporally coincided the increase in synaptophysin expression, as detected by Western blot (I, Fig. 2A). These results provided evidence that PPT1 activity associates with synaptic formation. These results also showed that PPT1 activity in neurons, especially in the retina, is under developmental regulation and that it increases with neuronal maturation.

1.2 PPT1 resides in axons in mature neurons

To analyse the exact intracellular distribution of PPT1 in neurons, localization of endogenous and Semliki Forest Virus (SFV) overexpressed PPT1 was studied by immunofluorescence in neurons at different maturation stages. In immature embryonic cortical stage 2 neurons cultured for two days no endogenous PPT1 was yet detected (I, Fig.3A). More mature stage 4 embryonic neurons, cultured for eight days, showed immunopositivity for endogenous PPT1 (red) that localizes mostly to MAP-2 negative axons (I, Fig.3B). In the postnatal neurons (postnatal day 1) cultured for 8 DIV, the MAP-2 negative neurites were intensively labeled for PPT1 (I, Fig. 3C). Moreover, the PPT1 positive puncta colocalized significantly well with axonal markers MAP-1 (I, Fig. 3D) and GAP-43 (I, Fig. 3E). Colocalization with GAP-43 was seen especially in axonal varicosities but less in growth cones (I, Fig. 3E). Mature embryonic retinal neurons showed a similar distribution (I, Fig. 3F). SFV overexpressed PPT1 distribution did not differ from the endogenous protein. In mature neurons the distribution of SFV-mediated human PPT1 colocalized with the presynaptic marker SV2 (I, Fig. 4A).

The expression of PPT1 preceded that of the synaptic vesicle protein 2 and synaptophysin, indicating that PPT1 has significance in the early development or maturation of neurons. To further specify the localization of PPT1 in axons, immunofluorescence analysis of PPT1 with the synaptic markers SV2 and synaptophysin was performed. Immunofluorescence staining of 8 DIV retina showed the endogenous PPT1 and SV2 distributed in the axonal varicosities (Fig. 5A). Endogenous PPT1 was detected in cortical neurons at a later time compared to retinal neurons, but in mature neurons PPT1 and SV2 were found abundantly in the presynaptic terminals (data not shown). Localization of PPT1 in axonal presynaptic terminals and associated with vesicular structures was also confirmed by double labeling with synaptophysin and postembedding immunoelectron microscopy (I, Fig.5B and 6A-F)

To quantify the axonal distribution of PPT1, mature hippocampal neurons were infected with a Semliki Forest Virus expressing recombinant human PPT1 and analysed by immunofluorescence (I, Fig. 4B). By using SFV-mediated expression, the start and duration of PPT1 expression could be controlled. SFV-mediated PPT1 intracellular distribution did not differ from endogenous. Quantitative measurements were performed in cells representing mature stage 4 to 5 neurons. Quantification resulted in over threefold higher pixel intensities in axons compared to dendrites ($P < 0.0001$; I, Fig. 4C) showing clear polarization of PPT1 distribution to axons.

This study showed a polarization of PPT1 distribution to axons in neurons. The specific localization of PPT1 in axons was further pinpointed to axonal varicosities with synaptic markers. The distribution data together with the high enzyme activity in mature neurons gave strong implications of the importance of PPT1 in functions outside the lysosomes in neurons. In addition, PPT1 was shown to precede mature synaptic markers in forming synapses and PPT1 activity was found to be under developmental regulation increasing upon neuronal maturation. These data together indicated that PPT1 is important both in the formation and the maintenance of synaptic contacts.

2. PPT1 deficiency causes endocytic defects connected to abnormal saposin processing (II)

Endocytic abnormalities have been shown to be present in non-neuronal cell types of neurodegenerative diseases (Cataldo, et al. 2000; Metzler, et al. 2001), classical lysosomal storage diseases (Chen, et al. 1998) and other diseases of the NCL group (Heine, et al. 2004; Luiro, et al. 2004). This study was initiated to clarify if such abnormalities were present also in INCL cells. The hallmark of the INCL disease is the accumulation of autofluorescent lipopigments, consisting of massive amounts of specific saposins A and D. The molecular mechanisms causing this have remained elusive. Prior to this study the localization of the saposin species had only been characterized on tissue level (Tyynelä, et al. 1993). The aims of this study were to point the exact intracellular localization of the saposin species in cells lacking PPT1, and to observe the fate of saposins along the endocytic pathway in INCL cells.

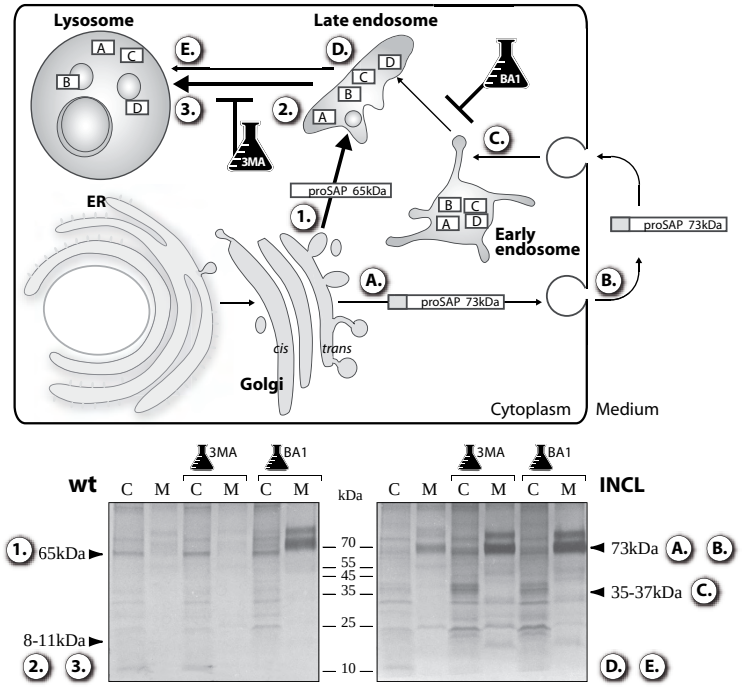
To investigate whether lack of PPT1 causes functional defects in the endocytic pathway, PPT1-deficient INCL patient fibroblasts were utilized. These cells were chosen because, even though INCL symptoms mainly arise from neuronal dysfunction, mechanisms of endocytic trafficking are relatively poorly understood in neurons. Furthermore, also non-neuronal cell types show storage deposits in the disease so defects were likely to be present as well. Nearing the end of this study the *Ppt1*^{Deltaex4} knockout mouse model became available and the subcellular localization of different saposin species was examined in the mouse neurons as well.

2.1 Delayed fluid-phase endocytosis in PPT1-deficient cells

Dissection of the function of the endocytic pathway was begun by studying the fluid-phase endocytosis (macropinocytosis, cell drinking) in the skin fibroblasts of INCL patients by following the uptake and endocytosis of a well characterized marker, fluorescent FITC-conjugated dextran (Shurety, et al. 1998; Swanson, 1989). The cells were fed with FITC-dextran and the uptake and timeing of transport along the endocytic pathway were analyzed using confocal microscopy. The degree of colocalization between the fluorescent marker and organelle-specific markers were quantified at various time points.

Colocalization of FITC-dextran with organelle specific markers EEA-1 for early endosomes and Lamp-1 for late endosomes/lysosomes was quantified at 10, 20, 40 120 min time points in 15 representative cells per cell type in three independent experiments. Co-localization values of normalized Pearson correlation coefficients in three

Figure 6. Saposin Maturation Pathways.



A representation of saposin biosynthesis and maturation pathways and consequences of endocytic inhibitor application.

The saposins are derived from a common precursor (proSAP, 65 kDa). The precurson has that has two alternative trafficking routes: Major pathway in wt cells comprises transport from the trans-Golgi network (1), through late endosomes (2) to lysosomes (3) cleavage occurring in late endosome and lysosomes. In the minor route the precursor is modified into a 73 kDa form (A) (proSAP, 73kDa) which is secreted (B) and traffics via the endocytic route (C,D) to lysosomes (E). Cleavage to four mature polypeptides (A, B, C and D, 8–11 kDa) occurs along the endo/lysosomal transport.

Bafilomycin A1 (BA1) blocks the transport from endosomes to lysosomes (C-D-E) and to some extent from the TGN to lysosomes (1-3). 3-methyladenine (3MA) blocks transport from late-endosomes to lysosomes (2-3, D-E).

In INCL cells, major part of intracellular proSAP was in the 73 kDa form (A). BA1 and 3MA treatments increased the secretion of the 73 kDa proSAP (B). Also an intermediate intracellular cleavage product of 35–37 kDa (C) was present in INCL cells and increased with BA1 and 3MA treatment.

combined experiments showed a statistically significant difference at 20 and 40 min time points in FITC-dextran localization between wt and INCL cells (Student's t test, $P < 0.05$) (II, Figs. 1A and B). FITC-dextran persisted in early endosomes in INCL cells showing a delay in fluid-phase endocytosis. Immunofluorescence stainings showed that after 10 min of chase, FITC-dextran colocalized well with an early endosomal marker EEA-1 in both wt and INCL fibroblasts. At the chase times of 20 and 40 min, in contrast, FITC-dextran was detected in late endosomes/lysosomes and not early endosomes in wt cells, (II, Figs. 1 C–D) but part of FITC-dextran was still retained in EEA-1 positive early endosomes in INCL cells (II, Figs. 1E–F). At 2 h of chase FITC-dextran was distributed in late endosomes/lysosomes in both wt (II, Figs. 1G–H) and INCL fibroblasts (II, Figs. 1I–J).

2.2 Delayed receptor-mediated endocytosis in PPT1-deficient cells

Receptor mediated endocytosis was investigated using two marker molecules: fluorescent low-density lipoprotein (BODIPY FL-LDL) and biotinylated transferrin. For BODIPY FL-LDL studies the fibroblasts were starved for lipoprotein to enhance marker uptake, and subsequently fed with BODIPY FL-LDL for 15 min. The cells were then fixed immediately or chased up to 40 min and analyzed using double immunofluorescence staining and confocal microscopy. Co-localization of BODIPY FL-LDL with organelle markers was quantified at indicated time points in 10 representative cells per cell type in three independent experiments. Colocalization values of normalized Pearson correlation coefficients in combined experiments showed a statistically significant difference between wt and INCL cells at all time points (Student's t test, $P < 0.05$) (II, Figs. 2A–B) showing delayed receptor-mediated endocytosis. In wt cells, fixed directly after 15-min LDL internalization, LDL had already passed the EEA-1 positive early endosomes and colocalized mainly with Lamp-1 in the late endosomes/lysosomes (II, Figs. 2C–D). However, in INCL cells, majority of the LDL colocalized with EEA-1 and only minority of the signal overlapped Lamp-1 at this time point (II, Figs. 2C–D). At 20 min of chase, LDL was detected in late endosomes/lysosomes in wt cells (II, Figs. 2G–H). In the INCL cells, LDL was also detected in lysosomes (II, Fig. 2J), however, some LDL still remained in the EEA-1

positive early endosomes (II, Fig. 2I). These data showed that also the receptor-mediated endocytosis is delayed in INCL cells.

Observed endocytic abnormalities were not caused by abnormal fusion or segregation of endocytic compartments in INCL cells, as immunofluorescence analysis of lysosomal marker aspartylglucosaminidase, AGA (Ikonen, et al. 1991) and endosomal marker EEA-1 showed that markers of different endocytic compartments in INCL cells segregate properly (II, Fig. 3A). Also fully functional endocytosis marker entry into the cells was verified by quantifying FITC-dextran and BODIPY FL-LDL uptake (II, Figs. 3B–C).

2.3 The recycling endocytosis pathway unaffected in PPT1-deficient cells

To examine the effect of different PPT1 expression levels on receptor-mediated endocytosis and the recycling pathway, endocytosis and recycling of transferring was investigated using biotinylated transferrin and a solid phase assay. Biotinylated transferrin was loaded into the cells at 17°C followed by a chase at 37°C to allow internalised transferrin to be recycled back into the extracellular space. The amount of biotinylated transferrin left inside the cells was measured at indicated time points using a quantitative ELISA assay.

To study the effects of PPT1-deficiency and over expression, transferrin recycling was compared in wt fibroblasts, stably transfected CHO cells (Lehtovirta, et al. 2001) and INCL patient fibroblasts. The patient cells exhibited no detectable changes in the rate of recycling when compared to wt (II, Fig. 4A). Protein palmitoylation has been shown to affect the recycling rate of the transferrin receptor. Alvarez and colleagues (Alvarez, et al. 1990) have reported a significant increase in the endocytosis rate of palmitoylation defective transferrin receptor as compared to the wt receptor. However, no difference in the rate of recycling could be detected in PPT1-overexpressing CHO-PPT1 cells when compared to wt CHO cells. CHO-PPT1 cells were also indifferent from CHO-AGA cell line over expressing another lysosomal enzyme aspartylglucosaminidase (II, Fig. 4B). To conclude, neither lack nor overexpression of PPT1 has an effect on the endocytic recycling pathway.

Similar abnormalities in specific endocytic pathways as observed here in INCL were also published in another NCL disease JNCL (*CLN3*), around the time of this study (Luiro, et al. 2004). Also in the variant form of LINCL (*CLN6*) defects in lysosomal degradation of arylsulfatase A marker were reported (Heine, et al. 2004). Interestingly the primary functions of these NCL proteins are proposed to be different and yet defects caused by the lack of these proteins result in similar effects on cell function. The exact reason for this is not entirely clear but one explanation might be that the NCL proteins have their normal function on a common cellular pathway. In fact, recent investigations have revealed molecular interactions between several NCL proteins, further implicating the presence of common mechanisms and possible shared pathways (Vesa, et al. 2002; Jalanko A., personal communication).

Growing body of evidence suggests that PPT1 has a function possibly in the regulation of certain intracellular membrane trafficking events. The protein's localization in places of active membrane transport in neural cells, the enzymatic depalmitoylation function a known regulator of trafficking events and here presented trafficking defect brought on by lack of the protein all point to this direction. The exact mechanism remains unclear however the trafficking defect present in PPT1 deficient cells most probably contributes to the neurodegenerative process in INCL.

2.4 SAPs A and D are redistributed in INCL fibroblasts and *Ppt1*^{Deltaex4} mouse knockout neurons

To elucidate the defective cellular pathway resulting in the specific accumulation of saposins A and D in INCL cells, the different saposin species were studied in wt and PPT1 deficient cells. Firstly, the subcellular distribution of proSAP and saposins A–D was studied by double immunofluorescence stainings and confocal microscopy in INCL fibroblasts and Ppt1 *Ppt1*^{Deltaex4} mouse primary hippocampal neurons. The distribution of both SAP A and D were strikingly different in INCL fibroblasts compared to wt fibroblasts. In wt cells, saposin D colocalized with a subpopulation of late endosomal/lysosomal vesicles identified by the markers Lamp-1 and LBPA (II, Figs. 5A–B), but not with EEA1-positive early endosomes (II, Fig. 5C). In INCL cells, SAP D showed a more abundant staining appearing relocalized throughout the cells co-localizing extensively with late-endosomes and also partially with early endosomes (II,

Figs. 5D, E, F). The localization of SAP B and C was similar in both wt and INCL fibroblasts, showing a colocalization with subpopulations of late endosomal/lysosomal Lamp-1 positive vesicles (II, Figs. 5G, H). Quantitation of the number of vesicles containing SAP A in wt and INCL cells showed a statistically significant increase of vesicles ($P < 0.05$) in INCL cells (II, Fig. 5I). Saposin A was also relocalized in *Ppt1*^{Deltaex4} mouse neurons, whereas saposin B showed a distribution indifferent from wt. In wt control cells, SAP A was localized in a subpopulation of late-endosomes/lysosomes as indicated by colocalization with Lamp-1 (II, Fig. 6A). In contrast, in the *Ppt1*^{Deltaex4} neurons, SAP A was partially localized in late-endosomes/lysosomes but was also seen in a more diffuse pattern appearing in other vesicular structures in the cell soma (II, Fig. 6B), in a similar manner to PPT1-deficient fibroblasts. The staining pattern of SAP B was same in control (II, Fig. 6C) and *Ppt1*^{Deltaex4} (II, Fig. 6D) neurons, showing a lysosomal distribution

2.5 Endocytic processing of saposins is abnormal in INCL fibroblasts

As abnormalities were found in transport of different markers along the endocytic pathway in INCL cells it was of interest to see is saposin processing was affected as well. The saposins are all derived from a common precursor (proSAP, 65 kDa) that has two alternative trafficking routes: it is either transported directly from the trans-Golgi network to the lysosomes or takes the alternative route in which case it is modified into a 73 kDa form, secreted and traffics via the endocytic route to lysosomes. In the endo-lysosomal compartment the precursor is proteolytically cleaved to the four mature polypeptides, SAPs A, B, C and D (8–11 kDa) (Fürst, et al. 1992; Hiesberger, et al. 1998; Sandhoff and Kolter, 1997). The details of the cleavage process, exact order of events, as well as the specific compartment in which it takes place, are still poorly characterized (Vielhaber, et al. 1996). The different saposin species were studied by metabolical labelling followed by immunoprecipitation in normal steady-state conditions and in the presence of pharmacological agents affecting endocytosis. Treatment with bafilomycin A1 (BA1) specifically inhibits vacuolar H+-ATPases resulting in the neutralization of endosomal pH, which blocks the transport from endosomes to lysosomes (Bowman, et al. 1988) and results in enhanced secretion of proSAP (Vielhaber, et al. 1996). 3-methyladenine (3MA) inhibits protein transport from late-endosomes

to lysosomes in a pH-independent manner (Punnonen, et al. 1994). The saposin maturation pathways and the effect BA1 and 3MA has on them are presented in figure 6.

In the wt cells, the proSAP was found mainly as a 65kDa form and only a small fraction of the precursor was found as a secreted 73kDa form (II, Fig. 7A). BA1 treatment increased the amount of the secreted 73kDa form of the precursor in wt cells (II, Fig. 7A). Interestingly, in the INCL fibroblasts, the intracellular proSAP was mostly found in the 73kDa form and hardly any 65kDa precursor was detected (II, Fig. 7B). Addition of BA1 or 3MA also increased the secretion of the 73kDa proSAP in INCL cells. In contrast to the wt cells, also the untreated INCL cells showed an increased amount of the secreted 73kDa proSAP (II, Fig. 7B). In addition, immunoprecipitation of the proSAP from the INCL cells revealed a presence of an unexpected doublet band of 35–37kDa in INCL cells (II, Fig. 7B). Treatment with BA1 or 3MA further increased the intensity of this intracellular band, but it was not found in culture the medium (II, Fig. 7B).

These studies show that there is an inherent shift of proSAP equilibrium to the indirect maturation pathway in the INCL cells, which is further enhanced by an endocytic inhibitor. Also the major part of proSAP in INCL cells is directed to the indirect maturation pathway. Increased secretion and endocytosis of proSAP has been reported from fibroblasts of I-cell disease patients that lack the ability to phosphorylate mannose residues (Fujibayashi and Wenger, 1986; Kornfeld and Mellman, 1989; Vielhaber, et al. 1996). Also, addition of the endocytic inhibitor bafilomycin, which alkalinizes the endosomal pH by 0.7–1.9 pH units (Presley, et al. 1997; van Weert, et al. 1997) has been reported to enhance the secretion of lysosomal enzyme precursors including prosaposin secretion by the neutralization of the pH of endosomes (Clague, et al. 1994; Presley, et al. 1997; van Weert, et al. 1997). Efficient uptake, processing and targeting of a number of secreted lysosomal enzymes, are heavily dependent on pH gradients. INCL fibroblasts inherently exhibit a bafilomycin-like effect as seen by the hypersecretion of proSAP. The pH in the lysosomes of INCL patient cells has been shown to be elevated (Holopainen, et al. 2001) and altered trafficking pathway of proSAP in INCL cells could be in part associated with the alkalization of endosomal pH. How-

ever, it is not the only reason leading to relocalization of saposins A and D and the abnormal processing of proSAP, since also the pH independent agent 3MA induced a similar effect in INCL cells. The sensitivity of proSAP processing to 3MA in INCL cells implies a defect in the vesicular fusion processes as 3MA is known to inhibit the fusion of late endosomes and lysosomes (Punnonen, et al. 1994). The abnormal processing of proSAP, and accumulation of the 35-kDa processing intermediate, may actually be the outcome of a delay in the processing of proSAP, supporting also the finding that endocytic events are delayed in INCL.

It is not clear why the maturation defect and subsequent relocalization and abnormal accumulation apply only to saposins A and D and not saposins B and C in INCL. Slowdown of endocytic trafficking in INCL cells could render the early maturation process of proSAP more vulnerable leading to abnormal cleavage intermediates. It could also result in an abnormal accumulation of the cleavage products on the endocytic pathway, explaining relocalization of saposins. It is possible that saposins A and D are more vulnerable to processing in earlier endocytic structures due to delays in endocytosis or changes in the pH, since they are located in the distal parts of the proSAP polypeptide chain.

This study established that lack of PPT1 causes specific endocytic defects in patient cells. Importantly, also abnormal saposin processing and relocalization was shown for the first time.

The exact nature of the endocytic defect, however, still remains unclear and further studies will be needed to characterize these events especially in neuronal cells. It will be of interest in the future to study why saposins A and D are specifically accumulated in INCL and not other NCLs, which also shows endocytic defects (Heine, et al. 2004; Luiro, et al. 2004). Studies will hopefully shed light on the detailed action of PPT1, saposins and other vesicular proteins and their contribution to the specific pathogenetic mechanism where the lack of PPT1 leads to neuronal death in INCL.

3. *Palmitoyl protein thioesterase 1 (Ppt1) deficient mouse neurons show alterations in cholesterol metabolism and Ca^{2+} homeostasis prior to synaptic dysfunction. (III)*

This study was started at a time when *Ppt1*^{Deltaex4} knockout mouse model had become available and preliminary studies on whole animal and tissue level had been initiated. The mouse had tissue pathology identical to the human counterparts but otherwise the phenotype seemed somewhat milder than in the human disease (Jalanko, et al. 2005). Events at the cellular level, especially in neuronal cells, needed clarification and functional studies commenced. Sensitive tools were needed for characterization of neuronal function and electrophysiological recordings, intracellular calcium homeostasis studies and different cellular stress inducing factors were chosen by virtue of their ability to show small dynamic changes in neuronal function. Also specific cellular metabolic processes were studied.

The genetic background of the INCL disease was discovered already in 1991 (Järvelä, et al. 1991), however, as is the case for many lysosomal storage disorders, the specific affected cellular and biochemical pathways and downstream secondary events have remained elusive. In order to find the early affected metabolic pathways global gene expression profiling combined with statistical and pathway analysis was utilized. Microarray studies were carried out with embryonic *Ppt1*^{Deltaex4} neuron and wt control neuron cultures.

3.1 Neuronal communication, maturation and lipid metabolism implicated in transcript profiling of *Ppt1*^{Deltaex4} neurons

Global gene expression analysis of approximately 14 000 known mouse genes in cultured cortical *Ppt1*^{Deltaex4} and wt control mouse neurons showed 135 statistically significant differentially expressed genes with false recovery rate of 1%. Of the identified genes, 106 were up-regulated and 29 down-regulated (III, Table 1.). The most up-regulated genes were synuclein α (*Snca*) with a fold change of +123 and adenylate cyclase-associated protein 1 (*Cap1*) with a fold change of +22. Cap1 is a capping protein associated with the actin cytoskeleton. Snca is localized at the presynaptic

sites in synaptosomes and has many functions in neurons including regulation of neurotransmitter secretion, maintenance of functional integrity of synaptic transmission, regulation of synaptic plasticity, synaptic vesicle transport and phospholipid metabolism (Golovko, et al. 2005; Liu, et al. 2004; Rochet, et al. 2004). *Snca* is up-regulated in many neurodegenerative disorders, including several diseases of the NCL group and has neuroprotective qualities (Leng and Chuang, 2006). The most down-regulated gene (-3,6) was *Shmt1*, encoding a soluble serine hydroxymethyl transferase that is critical for the synthesis of numerous cellular constituents required for cell growth.

Further analysis of the array data was carried out using Gene Ontology Tree Machine tool-kit according to Gene Ontology (GO) classifications (Ashburner, et al. 2000). The gene ontology analysis showed significantly more genes observed than expected ($P<0.01$) in 42 categories as follows: in biological processes three major pathways (III, Fig. 1 A), one molecular function (III, Fig. 1B) and in cellular compartments three distinct compartments (III, Fig. 1C). The affected biological processes were *cell communication*, specifically *secretion* and *transmission of the nerve impulse*, also *development*, specifically *neuron maturation* and *ensheathment*, and in *cellular metabolic process* the *lipid metabolism*. The molecular function with significant changes was binding, specifically calcium ion binding, lipid binding, identical protein binding and steroid binding (III, Fig 2.). The aspects of the implicated pathways were functionally analysed as described below.

3.2 Enhanced proliferative capacity of *Ppt1*^{Deltaex4} progenitor/stem cells

To understand early events in neuronal maturation and their potential consequences to INCL pathology, we studied neuronal stem cell maturation in culture. The proliferative capacity of the progenitor/stem cells as well as their maturation into various cell types was assessed. Proliferating neurospheres were dissociated and plated followed by a CyQuant Cell proliferation assay after 12 hours. In the high density experiments (initial 5000 cells per plate) the difference between *Ppt1*^{Deltaex4} and wt cells reached statistical significance (III, Fig. 3A). *Ppt1*^{Deltaex4} brain stem cells further exhibited normal differentiation capacity forming mature cell types present in the central nervous system: neurons (NeuN staining III, Fig. 3B), astrocytes (GFAP staining, III, Fig. 3C),

oligodendrocytes (GalC staining, III, Fig. 3D) and microglial cells (F4/80 staining, III, Fig. 3E). Neurite length was also studied as a marker for normal neuronal differentiation and was found to be similar in both wt and *Ppt1*^{Deltaex4} progenitor/stem cell derived neurons.

3.3 KO mouse neurons show fundamental changes in the cholesterol metabolism

Microarray studies showed upregulation in lipid metabolism, specifically in the metabolism of cholesterol and its derivatives and isoprenoid metabolism. Several cholesterol biosynthesis pathway key players, including the 3beta-hydroxysterol delta (14)-reductase acting at late steps of cholesterol biosynthesis, were upregulated. These findings prompted us to study cholesterol biosynthesis and steady state cholesterol content in *Ppt1*^{Deltaex4} mouse neuron cultures compared to wt neurons.

The changes seen in microarray analysis did manifest on the functional level in neuron cultures. The rate of sterol synthesis was found to be upregulated *Ppt1*^{Deltaex4}neurons compared to wt. Especially the conversion of sterols into the end product cholesterol and its immediate precursor desmosterol was markedly upregulated in *Ppt1*^{Deltaex4}neurons (III, Fig. 4A). When total sterol amounts were analyzed, we found that cholesterol was unchanged in *Ppt1*^{Deltaex4} neurons as compared to wt but the amounts of its immediate precursors were reduced (III, Fig. 4B). This result agrees with the enhanced conversion of precursor sterols into the end product as observed with radiolabeled sterols. The amount of unlabeled lanosterol was also reduced in the *Ppt1*^{Deltaex4} neurons. This tendency is in line with the upregulation of 3β-hydroxysterol Δ(14)-reductase mRNA as this is a post-lanosterol enzyme in sterol biosynthesis. These findings may reflect the leakage of cholesterol out of the cells into the growth medium. While the exact molecular mechanisms underlying these changes remain unclear they may have fundamental effects on neuronal function. Defects in cholesterol metabolism have been implicated in neurological defects in several diseases (Vance, Hayashi, and Karten, 2005). In the Niemann-Pick type C (NPC) disease impaired cholesterol transport causes neurological symptoms resulting from accumulation and cellular imbalance of cholesterol. The imbalance is manifested in neurons by reduced cholesterol content in the axons causing synaptic dysfunction. It has also been reported that NPC fibroblast cells have traffick-

ing defects leading to increased cholesterol synthesis and expression of LDL receptors. (Reviewed in Liscum and Sturley, 2004) Impairments in intracellular trafficking are also shown in INCL fibroblasts in this thesis work and earlier also in other NCL fibroblasts (Ahtiainen, et al. 2006; Luiro, et al. 2004). These include slowdown in the transport rate of labelled LDL in the endocytic route. If this defect is also present in neuronal cells it might have an impact on cholesterol homeostasis.

Cholesterol is also a regulator of neuronal development being essential for axonal growth and repair (Posse De Chaves, et al. 2000; Trommsdorff, et al. 1999). Abnormalities present in the cholesterol metabolism may have developmental implications in INCL. Amount and ratio of cholesterol and it's precursors change the fluidity of the membrane in which they reside (Vance, et al. 2006). Changes in this balance possibly affect developmental processes in neurons such as axonal extension. As cholesterol is important in spatial organization, fusion and recycling of synaptic vesicles (Jia, et al. 2006; Jeremic, et al. 2006), another possibility is that the increased cholesterol biosynthesis in *Ppt1*^{Deltaex4} neurons serves to preserve functional synapses. The upregulation of cholesterol biosynthesis may by a secondary phenomenon resulting from the primary Ppt1 deficiency. However, it is possible that the changes observed here in the sterol balance of *Ppt1*^{Deltaex4} neurons may reflect compensatory mechanisms to preserve raft integrity and functions since altered levels of PPT1 expression have previously been associated with altered lipid composition of rafts (Goswami, et al. 2005).

3.4 KO mouse neurons recover more readily from glutamate induced Ca^{2+} release

Defects in maintenance of intracellular calcium homeostasis have been previously reported in neurodegenerative disorders (Albin, 2003; Etcheberrigaray, et al. 1998; Ho, et al. 2001; Mandel, et al. 2003). Observing maintenance of calcium homeostasis is a sensitive tool to study subtle dynamical changes in neuronal function. Microarray analysis of *Ppt1*^{Deltaex4} mice also showed significant changes in transcripts encoding calcium binding proteins of which significantly up-regulated are shown in Table 2 (III, Table 2).

To test functional properties of *Ppt1*^{Deltaex4} mouse neurons primary embryonic cortical and hippocampal mixed neuron cultures were utilized for the study in intracellular Ca^{2+} homeostasis using the calcium indicator Fura-2. Wt and KO cultures accumulated the dye with similar efficiency. Both cultures responded similarly to depolarization induced by 50 mM K^+ (Fura-2 340/380 ratio: 2.9±0.3 *versus* 3.4±0.3, n=70 and 96 cells in KO and wt, respectively; P>0.05). After full recovery of Ca^{2+} back to the pre-stimulation baseline, cells were challenged with 100 μM glutamate. Although glutamate-induced peak Ca^{2+} levels were similar between wt and *Ppt1*^{Deltaex4} cells, post-glutamate recovery of Ca^{2+} to the baseline was different in that *Ppt1*^{Deltaex4} cells recovered more efficiently that their wt counterparts. To quantify these results, we analysed glutamate responses in cells that responded to the initial stimulation with elevated K+. Among the cells that responded to glutamate, 47±11% of *Ppt1*^{Deltaex4} cells returned to their pre-stimulation Ca^{2+} levels, while only 27±9% of wt cells were able to recover to baseline Ca^{2+}. At the end of each experiment, culture dish was perfused with the ionophore ionomycin (50 μM), and the 340/380 ratio corresponding to the maximal Ca^{2+} level was found to be similar between *Ppt1*^{Deltaex4} and wt cells (5.6±0.5 and 6.2±0.6, respectively; P>0.05). Thus, despite similar properties of basic Ca^{2+} homeostasis, *Ppt-1*^{Deltaex4} cells exhibited a tendency to improved recovery after a potentially excitotoxic stimulation with glutamate, as compared to wt cells.

The reason for this tendency for more efficient recovery exhibited by *Ppt1*^{Deltaex4} neurons is not altogether clear but a contributing factor may be the up-regulation of several transcripts encoding calcium binding proteins, found in the microarray analysis of KO mouse neuron cultures. Some of these calcium binders are directly associated with protection of glutamate induced cytotoxicity such as calbindin1. Also other less specific factors reported to have neuroprotective activities, such as synuclein alpha, were also up-regulated in the arrays possibly providing additional protection from activity induced stress and cytotoxicity. It has been proposed that ER stress mediated unfolded protein response would lead to impairment in the maintenance of calcium homeostasis in INCL. ER stress, possibly caused by accumulation of palmitoylated substrates, would also induce reactive oxygen species (ROS) increase leading to mitochondrial membrane dysfunction and following caspase-9 activation and apoptosis. (Kim, et al. 2006b). We could not, however, find implications of mitochondrial dysfunction or increased ROS nor did we detect increase in apoptosis in cultured

neuronal cells (data not shown). One explanation for the observed efficient recovery and resistance to cytotoxicity could be that KO cells are less responsive to glutamate induction *per se*, possibly due to decrease in the number of glutamate receptors or their activity. However, this does not seem to be the case since peak levels of released calcium at induction are similar in *Ppt1*^{Deltaex4} and wt cells. Also glutamate receptor abnormalities would also be expected to be manifested in defects in synaptic function. However synaptic function is unaffected as shown in following.

3.5 *Ppt1*^{Deltaex4} hippocampal CA1 neurons have unaltered active electrophysiological properties in young mice

The microarray analysis results strongly pointed to changes in factors that affect synaptic function including significant changes in gene categories encoding factors that regulate neurotransmitter secretion and neurotransmitter levels, synaptic transmission and action potential regulation, nerve ensheathment and myelination. To assess whether lack of Ppt1 has an impact on synaptic function we studied the active electrophysiological properties of *Ppt1*^{Deltaex4} mouse neurons in acute brain slices in the CA1 region pyramidal neurons. The *Ppt1*^{Deltaex4} neurons exhibited unchanged basic conductive properties as indicated by similar synaptic delay values in wt and *Ppt1*^{Deltaex4} neurons (wt 5.5ms ± 1.1, n=8; KO 6.9ms ± 0.8, n=8; P>0.05). Also the properties of stimulation-induced EPSPs amplitude, the slope, rise and decay times were similar (III, Fig. 5A).

To characterize efficacy of synaptic transmission, stimulation-response curves obtained from wt and *Ppt1*^{Deltaex4} neurons. Schaffer collaterals were stimulated with intensities ranging from 2mV to 30mV and the amplitude of synaptic response was measured. Wt and *Ppt1*^{Deltaex4} neurons showed identical stimulation-response relation (III, Fig. 5B). They had a similar threshold for initial response and a similar slope of the stimulus-response curve. Furthermore, the largest possible response did not differ in wt and *Ppt1*^{Deltaex4} neurons (III, Fig. 5 B).

Properties of short-term synaptic plasticity between *Ppt1*^{Deltaex4} and wt neurons were compared by measuring paired-pulse responses with increasing inter-stimulus intervals. No differences could be found in short term plasticity measured by paired pulse

responses in wt and *Ppt1*^{Deltaex4} neurons (III, Fig. 5 C). The rate of depletion of the readily releasable pool of synaptic vesicles during repeated stimulation was studied by tetanic stimulation causing progressive depletion of the synaptic vesicle. Both Wt and *Ppt1*^{Deltaex4} neurons responded similarly to repeated stimulation. The plateau of 109.6pA (± 28.6, n=11) for wt and 125.2pA (± 38.2, n=8) for *Ppt1*^{Deltaex4} were reached at 267ms (±27) and 261ms (±27) respectively (III, Fig. 5 D)**.**

Based on the thorough analysis of 16 to 25 day old mouse hippocampal CA1 region pyramidal neurons it is clear that lack of Ppt1 does not affect the active electrophysiological properties of this particular neuron type at this time point. Previous findings on the mouse model and also data on the patients suggest that even though storage material is present in a very early time point, even prenatally, gross changes in neuronal function and neuronal death start at a later time. The mice show loss of vision not until the age of 8 weeks, seizures after 4 months and paralysis of hind limbs at the age of 5 months (Jalanko, and Vesa, et al, 2005). So it likely that regulation of neuronal function is so crucial, that compensatory mechanisms mask the events of early pathogenesis in young animals. This is also supported by the findings in the other *Cln1*$^{-/-}$ mouse model: The *Ppt1*$^{tm1Hof-/-}$ mouse cultured embryonic neurons have increased lysosomal pH, normal synaptic density but reduced number of readily releasable pool of synaptic vesicles. In spite of this, they exhibit unchanged active electrophysiological properties (Virmani, et al. 2005). Also a contributing factor, to the unchanged electrophysiological findings reported in this study, may be a differential selective pattern of neurodegeneration in the hippocampus: INCL patients have been reported to have significant degeneration in the CA2, CA3 and CA4 regions of the hippocampus whereas the CA1 region is relatively spared (Tyynelä, et al. 2004). Neuroanatomical studies of the *Ppt1*$^{tm1Hof-/-}$ mice implicated that the selective neuronal loss in INCL most dramatically influences the somatosensory cortex and thalamus (Bible, et al. 2004). It is also plausible that initiation neuronal death in the disease has a threshold; cells are able to tolerate and compensate cellular defects up to a certain point, after which pathogenetic mechanism take over. This can further be inferred from the fact that non-neuronal cells are mostly not affected in INCL even though cellular defects, such as delays in specific endocytic pathways and abnormalities in storage protein metabolism, are present in them too (Ahtiainen, et al. 2006). Neurons

may be more vulnerable to these defects due to their restricted regenerative potential and more complex cellular metabolism.

The specific mechanisms of neuronal death present in INCL still remain unsolved. The selective pattern of neuronal loss implies the presence of specific mechanisms and in fact increased caspase cascade mediated apoptosis has been reported on *Ppt1*$^{tm1Hof-/-}$ mice and patient tissue (Zhang and Kaufman, 2006). However, while cultured *Ppt-1*^{Deltaex4} embryonic show definite changes in several cellular functions, no increase is observed in the rate of apoptosis nor necrosis in steady state or cellular stress inducing conditions (Kopra O., unpublished observations). Moreover, connections to regulation of autophagic processes arise from the fibroblast studies (Ahtiainen, et al. 2006): alterations in fluid-phase endocytosis and effects of the autophagic inhibitor 3MA on metabolism in PPT1 deficient cells. Recent studies have associated autophagic events to apoptotic cell death (Guillon-Munos, et al. 2006; Yu, et al. 2004). As many key players are shared by these pathways it is plausible that different modes of cell death also act in concert in INCL.

This study established that lack of Ppt1 has fundamental effects on neuronal function. Ppt1 is crucial in neuronal maturation and development and also in the function of mature neurons. Lack of Ppt1 has an impact on many cellular functions directly or indirectly including lipid metabolism, maintenance of calcium homeostasis and early proliferation. These studies showed for the first time that Ppt1 deficiency causes fundamental defects in neuronal cholesterol biosynthesis and the connection between NCL and lipid metabolism deserves more attention in the future.

Conclusions and future prospects

When this study was initiated the genetic background of the NCL disorders had been elucidated to a large extent, through identifying genes behind the diseases and a number of disease causing mutations. However the biological processes and functional defects underlying disease mechanisms were still poorly understood. The focus of research shifted towards better characterizing early events in disease progression through functional and cell biological approaches. The first approaches utilized patient cell models. However, major advances in the understanding the molecular basis of NCLs, came through the emergence of genetically engineered mouse disease models. In recent years, thorough characterization of the mouse models has revealed new aspects of disease neuropathology and defects in cellular processes underlying neurodegeneration have started to unfold. Also utilizing system wide approaches and high-throughput methods has provided new avenues of research and advances have also been made in discovering potential therapeutic approaches. In spite of these major advances, still fairly little is understood of the connections of primary enzyme defects leading to storage of intracellular material and the following downstream effects. Therefore the impact the intracellular storage on neuronal function is only beginning to be understood.

The major findings in this thesis work include the characterization of PPT1 localization in mature synapses and expression patterns in early neuronal maturation stressing the importance of PPT1 to neuronal maturation as well as mature neuronal function. Furthermore we present the first connection of intracellular storage material to defects in endocytic transport in patient cells. Early events underlying disease pathology were discovered through high-throughput methods, and confirmed in functional studies of the Ppt1 deficient mouse disease model. These defects included changes in early maturation and impact on cholesterol and calcium metabolism. Similar defects have been found to contribute to pathology in other LSDs as well as neurodegenerative disorders (Korkotian, et al. 1999, Lloyd-Evans, et al. 2003; Pelled, et al. 2003; Jeyakumar, et al. 2005). Further characterization and better understanding of the molecular basis and the exact mechanisms of these defects will be of utmost importance in future studies.

Thorough tissue level neuropathological and neuroanatomical investigations on the disease model mice have revealed specific patterns of neuronal death in NCLs. The cortical neurons are most affected in the NCLs, however there are prominent differences between the severity of neurodegeneration in different cortical layers and furthermore between resident neuron populations. The first signs of neurodegeneration are present, not in the cortical area, but in the thalamic region (Bible, et al. 2004; Pontikis, et al. 2004; Weimer, et al. 2006). These findings have lead to reassessment of the sequence of events leading to neuronal death in the NCLs. The thalamus has an important role as a relay center for sensory inputs to the cerebral cortex from the periphery. The cortical lamina IV pyramidal neurons in the primary sensory cortices receive input from the thalamus and these cells are severly affected in the INCL mouse model. It is plausible to assume that neuronal connections play a role in INCL pathology. In addition, loss of inhibitory GABAergic interneurons in several brain areas implies defects in maintenance of the balance of inhibitory and excitatory inputs contributing to neuronal dysfunction and death (Gupta, et al. 2001; Jalanko, et al. 2005). Localization and identity of the neuron are central determinants of neuron survival in INCL and calcium related functions and their alterations are a strong candidate in this process (Das, et al. 1999; Haltia, et al. 2001; d'Orlando, et al. 2002; Cooper, 2003; Kim, et al. 2006; Cooper, et al. 2006). Further studies defining processes, that render specific neuronal populations more vulnerable, are needed. Some controversy exists on neuronal death mechanisms in INCL. Suggested models include apoptosis and autophagy (Mitchison, et al. 2004). Of these, ER stress induced apoptosis described in the mouse model as well as patient autopsy samples, is best characterized (Zhang and Kaufman, 2006). It is probable that several mechanisms apply depending on the temporal and spatial context during disease progression. Delineating the specific mechanisms of neuronal death, present in different neuron populations, will broaden the understanding of the cascade of pathogenetic events in the disease.

Growing evidence suggests that developmental issues, rather than just degenerative aspects, are central contributors to INCL disease pathology. Interactions of neural and glial cells in developing brain and in regulation of mature neuronal function may turn out to be central in INCL. Glial cells provide and maintain the local chemical environment necessary for proper neuronal function. Pervasive astrocytic and more limited microglial activation are concurrent and even precede neurodegeneration in

INCL (Elshatory, et al. 2003). Glial activation demarcates abnormal inflammatory responses, especially in later stages of disease progression, but glia may also play a role in early pathogenetic events. The role of lipid and calcium metabolism in neurons needs to be studied from the developmental viewpoint and also in relation to glial responses. Furthermore, analysis of PPT1 in relation to other developmental players and synaptic proteins as well as other NCL proteins, utilizing high-throughput and *in vivo* functional methods, are essential understanding the events leading to neuronal degeneration and ultimately death in INCL. These studies will provide information leading to understanding of the exact function and targets of PPT1 action *in vivo*.

Acknowledgements

This study was carried out at the Department of Molecular Medicine, National Public Health Institute during 1999-2007. The former and present Directors of the Institute, Jussi Huttunen and Pekka Puska and Heads of the Department Leena Palotie, Christian Ehnholm and Anu Jalanko are acknowledged for providing excellent research facilities. I also wish to thank Docent Pekka Lappalainen, Anita Tienhaara and Erkki Raulo from the Graduate School in Biotechnology and Molecular Biology.

I wish to express my gratitude to Professor Jim Schröder for accepting the role of *kustos* at my thesis defense. Professor Pertti Panula and Dr Jussi Jäntti are thanked for reviewing and improving the manuscript of this thesis. Professors Pertti Panula and Elina Ikonen are thanked for a valuable and fruitful thesis committee meeting. I wish to express my appreciation to Professor Anu Wartiovaara for accepting the role of opponent at my thesis dissertation.

This thesis has been supervised by Docent Anu Jalanko and Docent Outi Kopra. I joined Anu's research group in 1999 in the summer Anu later described "I have no idea what you girls were doing while I was away that summer, but it must have been good since you all stayed." These years have been a remarkable journey with no shortage of unforeseeable turns. I wish to thank you Anu for accompaniment through the ups and downs of science. Rest assured I have grown as a researcher and a human being during these years. I value the independence you have granted me from the start as well as the trust you have expressed in my abilities. I wish to thank you Outi for treating me as an equal partner in science and for solidarity when we were having the wind against us. Your expertise in the life and death of neurons has been invaluable.

I wish to express gratitude for all of my co-authors and collaborators: Minna Laine for first lessons in the lab with AGU mice, Jaana Tyynelä for the expertise with saposins, Aija Kyttälä for critical comments and help in improving the second publication, Max Gentile for expert help with the microarray data, Leo Khiroug for guidance into the world of electrophysiology and calcium imaging studies, Professor Elina Ikonen and Aino-Liisa Mutka for smooth and productive collaboration with the cholesterol studies.

I wish to thank past and present members of the AGU-NCL group: Kaisu, Tintti, Annina, Kristiina, Tarja, Liina, Salli, Minna, Kaitsu, Juha, Ville and Jani for the valuable experience and creating a pleasant working atmosphere. Above all my office mates and fellow NCL'ers Kaisu, Tintti, Annina and Kristiina are thanked for great company. I would also like to thank the technicians that have worked in the group, Anne, Auli, Kaija, Marja, Seija and Tuula for expert assistance.

The coffee collective - I would have been lost without you. Thanks for all the years in the same boat! Kaisu, Tanja, Maria, Taina, Nora, Anna, Liina and Heidi have been great friends, fellow researchers and invaluable support home and abroad. Iski is acknowledged for solid insights in matters between the earth and the sky.

Over the years many exceptional people have tread though the lab. Dear colleagues, people of the “big lab” senior and junior, secretaries Sari K, Sari M and Tuija, computer support Jari, thanks for everything. Marjo Kestilä is especially thanked for help with the final stage bureaucracy in publishing this thesis.

Friends and family are thanked for encouragement and affection and all the good things in life.

Lasse, Lumi and Liina, you mean the world to me.

In Helsinki, April 2007

Laura

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