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MOLECULAR AND CELLULAR BIOLOGY OF INFANTILE NEURONAL CEROID LIPOFUSCINOSIS (INCL)

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Academic Dissertation

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

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

I Salonen T, Järvelä I, Peltonen L and Jalanko A. (2000): Detection of eight novel palmitoyl protein thioesterase (PPT) mutations underlying infantile neuronal ceroid lipofuscinosis (INCL; CLN1). Human Mutation 15:273-279.

II Salonen T, Heinonen-Kopra O, Vesa J and Jalanko A (2001): Neuronal trafficking of palmitoyl protein thioesterase provides an excellent model to study the effects of different mutations which cause infantile neuronal ceroid lipofuscinosis. Molecular and Cellular Neuroscience 18:131-140.

III Salonen T, Hellsten E, Horelli-Kuitunen N, Peltonen L and Jalanko A. (1998): Mouse palmitoyl protein thioesterase: Gene structure and expression of cDNA. Genome Research 8:724-730.

ABBREVIATIONS

aa	amino acid
ANCL	Adult neuronal ceroid lipofuscinosis
BHK cells	Baby hamster kidney cells
bp	base pair
cDNA	complementary deoxyribonucleic acid
CLN1	infantile neuronal ceroid lipofuscinosis locus
CLN2	classical late infantile neuronal ceroid lipofuscinosis locus
CLN3	juvenile neuronal ceroid lipofuscinosis locus
CLN4	adult neuronal ceroid lipofuscinosis locus
CLN5	variant late infantile neuronal ceroid lipofuscinosis locus, Finnish type
CLN6	variant late infantile neuronal ceroid lipofuscinosis locus
CLN7	variant late infantile neuronal ceroid lipofuscinosis locus, Turkish type
CLN8	Northern epilepsy locus
CONL	congenital ovine neuronal ceroid lipofuscinosis
COS-1 cells	African green monkey kidney cells
DNA	deoxyribonucleic acid
EEG	electroencephalogram
ER	endoplasmic reticulum
ES cells	embryonic stem cells
FISH	fluorescent *in situ* hybridization
GROD	granular osmiophilic deposits
INCL	infantile neuronal ceroid lipofuscinosis
JNCL	juvenile neuronal ceroid lipofuscinosis
kb	kilobase pairs
kDa	kilodalton
LINCL	late infantile neuronal ceroid lipofuscinosis
mRNA	messenger ribonucleic acid
NCL	neuronal ceroid lipofuscinosis
NSF	N-ethylmaleimide-sensitive fusion protein
OMIM	online Mendelian inheritance in man
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PPT1	palmitoyl protein thioesterase 1
PPT2	palmitoyl protein thioesterase 2
RNA	ribonucleic acid
SNAP	soluble NSF attachment protein
SNARE	SNAP receptor
TPP1	tripeptidyl petidase 1
VAMP	vesicle associated membrane protein
wt	wild-type

In addition, standard one-letter abbreviations of nucleotides and three-letter abbreviations of amino acids are used.

SUMMARY

Infantile neuronal ceroid lipofuscinosis (INCL) is a severe, recessively inherited, progressive encephalopathy of children, which is part of the Finnish disease heritage. INCL is the early-onset form of neuronal ceroid lipofuscinosis, a disease group consisting of at least eight neurodegenerative storage disorders characterized by accumulation of ceroid and lipofuscin like deposits. The worldwide incidence of NCL disorders is estimated to be about 1 in 20 000 births. INCL is caused by a deficiency of the palmitoyl protein thioesterase 1 (PPT1) enzyme.

Several mutations in the PPT1 gene have been described and we have here characterized eight novel PPT1 mutations in patients of diverse ethnic origin. PPT1-deficient patients with a disease resembling late infantile and juvenile NCL have been described earlier. The identification of mutations was followed by analysis of a selected set of mutations resulting in classical early or protracted juvenile like disorder. All the mutated polypeptides were located in the endoplasmic reticulum of transiently transfected BHK cells. The intracellular maturation of the mutated PPT1 polypeptides was also analyzed in nonneuronal cells but no difference from the wild-type PPT1 could be demonstrated. Furthermore, the enzymatic activity of the mutant polypeptides was similarly reduced, despite the observed differences in the patients' phenotypes. The trafficking of the mutant PPT1 polypeptides was also monitored in neuronal cells, using Semliki Forest virus-mediated expression. A remarkable difference was demonstrated between the mutations causing the mild and the severe phenotypes. Polypeptides associated with severe phenotype were retained in the ER, but those with milder phenotype migrated along the neuritic shafts similarly to the wild-type PPT1. Thus, for the first time, a phenotype-genotype correlation in INCL was demonstrated at cellular level. This result indicates that neuronal cell models are indispensable in the analysis of molecular trafficking and disease mechanism in NCL and similar neurological disorders. The third study included in the thesis describes the cloning, chromosomal location, and genomic structure of the mouse PPT1 gene. The transient expression of the 38/36 kDa polypeptide and its lysosomal localization in peripheral cell lines was demonstrated. The degree of homology between mouse and human PPT1 is 96%, indicating the high conservation of this gene. The characterization of the mouse PPT1 gene provides a basis for the production of a knock-out mouse model which should provide further opportunities to study the disease mechanism of INCL.

REVIEW OF THE LITERATURE

1. Neuronal ceroid lipofuscinoses

1.1 The Finnish disease heritage and neuronal ceroid lipofuscinoses

Archeological studies show that Finland has been inhabited since the last glacial period, over 11 000 years ago. However, these earliest inhabitants were not the ancestors of today's Finns. According to the dual theory, Finland was settled by two small groups of founders: the first migration wave, by eastern Uralic speakers from Russia, took place some 4000 years ago and the second, by immigrant populations coming across the Gulf of Finland about 2000 years (or 80-100 generations) ago (Nevanlinna, 1972) (de la Chapelle, 1993). On the basis of maternal mitochondrial and paternal Y-chromosomal analyses, today's Finns have been shown to be descendents of this latter group (Sajantila et al., 1996; Kittles et al., 1998). For geographical, cultural, and religious reasons, interactions with the neighboring countries were rare, leading to isolation. The number of original founders was probably only a few hundred or thousand individuals (de la Chapelle, 1993). Until the 16th century, only the southern and western coasts of Finland, south Savo, and the region east of Lake Ladoga were inhabited (the so-called early settlement). By this time, the population is estimated to have been 250 000. In consequence of population expansion, migration began toward the central, northern and eastern parts of Finland began (the so-called late settlement). Typically, some 20-40 immigrant families formed a community, and these may have remained separated for centuries on account of the sparse population and the long distances (Nevanlinna, 1972; Norio et al., 1973; de la Chapelle, 1993; Peltonen et al., 1999). The expansion of the Finnish population has occurred during the last three centuries, from 250 000 at the beginning of the 18th century to today's 5 100 000 inhabitants.

The concept of the Finnish disease heritage was initially described in 1973 by Norio, Nevanlinna, and Perheentupa (Norio et al., 1973). Today, this disease group includes 35 diseases mostly inherited recessively, which are more prevalent in Finland than in other populations. The number will most likely be higher in the future, since new genetic diseases are still being discovered (Fellman et al., 1998). It has been estimated that 15% of Finns are carriers of mutations belonging to the Finnish disease heritage. On the other hand, the incidences of several hereditary diseases which are common in other populations, such as phenylketonuria, cystic fibrosis, and Huntington's disease, are strikingly lower in Finland and therefore the number of genetically affected patients is not higher in Finland than in other countries (Kere et al., 1989) (Palo et al., 1987). Some of the disease genes carried by the small group of original founders (the founder effect) were lost and others were enriched as a result of genetic drift (random fluctuations in gene frequencies). Consanguineous marriages increased the enrichment of rare recessive disorders. The genetic homogeneity, the accurate church record system covering the period until 1640, and the high quality of the health care system

provide an excellent basis for genetic research in Finland. The geographical distribution of the ancient mutations, which were present in the initial founder population, follows the routes of migration. The classical storage disorder causing aspartylglucosaminuria is an example of an ancient mutation (Norio, 1981).

On account of the long isolation period, the Finnish gene pool is homogeneous. Therefore the diseases of the Finnish heritage are each typically caused by one major mutation, as is the case in infantile neuronal ceroid lipofuscinosis, in which 98% of the Finnish patients are homozygous for the same mutation ($INCL_{Fin}$) (Vesa et al., 1995). This mutation is estimated to have been introduced 40 generations ago into the subpopulation living along the western coastline of Finland. The geographical distribution of patients with INCL mainly resembles the present-day population density, but a clustering of cases in the western coastal area of Finland can still be observed (Peltonen et al., 1995). In the younger mutations, regional clustering can still be clearly observed. For example, the Finnish type of variant late infantile neuronal ceroid lipofuscinosis was introduced into Southern Ostrobothnia 500 years (20-30 generations) ago (Varilo et al., 1996). In total, about 400 patients in Finland have been diagnosed as suffering from NCL disorders. The most common types are JNCL with 185 cases and INCL with 158 cases (Santavuori et al., 2000).

1.2 Historical background

The first description of patients with the clinical features of neuronal ceroid lipofuscinosis (NCL) was given by Stengel in 1826. He reported four siblings with epilepsy, motor dysfunction, dementia, and progressive blindness (Stengel, 1826). To classify disorders of a similar type, consisting of progressive psychomotor deterioration and blindness, Sachs termed these diseases as amaurotic familial idiocy (AFI); also known as Tay-Sachs disease (Sachs, 1896). Patients with clinical findings similar to those reported by Stengel were described by Batten in 1903 (Batten, 1903), by Spielmeyer in 1905 (Spielmeyer, 1905), by Vogt in 1905 (Vogt, 1905) and by Sjögren (Sjögren, 1931) and patients with a more rapidly developing clinical phenotype were detected by Jansky (1908) (Jansky, 1908) and Bielschowsky (1913) (Bielschowsky, 1913). Finally, an adult form of the disease was described by Kufs (1925) (Kufs, 1925). The clinical variation between the AFI cases was wide, but all the patients had neuronal loss and accumulation of lipid material in remaining swollen nerve cells. In 1962, Svennerholm reported that the storage material in classical Tay-Sachs disease is composed of gangliosides (Svennerholm, 1962). In 1963, Zeman and Alpert reported that the intraneuronal storage material in their patients suffering from variant AFI was fluorescent and the tinctorial properties resembled ceroid and lipofuscin (Zeman and Alpert, 1963). The term neuronal ceroid lipofuscinosis was introduced by Zeman and Dyken in 1969 to distinguish these disorders from Tay-Sachs disease (Zeman and Dyken, 1969).

Lipofuscin (also known as age pigment) is an electron-dense, autofluorescent material that normally accumulates with aging in all postmitotic cells, such as neurons. It is

mainly composed of mixtures of proteins and lipids. Ceroid is a very similar or probably identical material to lipofuscin, but it accumulates under pathological conditions, such as, lysosomal storage diseases and tumors (Terman and Brunk, 1998).

1.3 Original classification

NCLs are progressive neuronal disorders, which are distributed worldwide and are the most common neurodegenerative disorders in children. The estimated carrier frequency is about 1% world wide, but the incidence of the different NCL subtypes in different populations is extremely variable. In Finland and in the USA the estimated incidence is 1:12 500 (Rider and Rider, 1988; Santavuori, 1988). On the basis of age of onset, clinical symptoms, neurophysiological findings, and ultrastructure of the storage material, four major subtypes of NCL are recognized: Infantile NCL (INCL, gene locus CLN1), late infantile NCL (LINCL, CLN2), juvenile NCL (JNCL, CLN3), and adult NCL (ANCL, CLN4) (Table 1).

	Infantile NCL	Late Infantile NCL	Juvenile NCL	Adult NCL
Synonyms	Santavuori-Haltia	Jansky-Bielshowsky	Spielmayer-Vogt-Sjögren Batten	Kufs' Parry
Age of onset	8-18 months	2-4 years	5-10 years	10-55 years
First symptom	Psychomotor retardation	Epilepsy	Visual failure	Dementia
Ultrastructure of storage material	GROD	Curvilinear	Fingerprint	Fingerprint + GROD

Table 1. Classification of the four major subtypes of NCL. GROD = granular osmiophilic deposits.

Later on, many atypical cases that do not fall into any of these four groups have been identified (Carpenter et al., 1973; Lake and Cavanagh, 1978; Santavuori et al., 1982; Wisniewski et al., 1988; Santavuori et al., 1991; Dyken and Wisniewski, 1995; Hirvasniemi et al., 1995; Hofman and Taschner, 1995; Elleder et al., 1997; Sharp et al., 1997; Mitchison et al., 1998; Wisniewski et al., 1998; Williams et al., 1999c). According to current knowledge, at least eight, genetically different, NCL groups exist and these disease loci have been named from CLN1 to CLN8 (Table 2).

1.4 Storage materials

Each of the main NCL subtypes has storage material with a characteristic ultrastructure, and these have been used to classify the NCL disorders into three main subtypes. INCL patients accumulate storage material characteristic of granular osmiophilic deposits (GROD)(Fig 1). These particles are finely granular membrane-bound bodies with diameters of 0.5 μm and may form aggregates up to 5 μm in diameters (Haltia et al., 1973a; Haltia et al., 1973b). When the accumulating storage material in INCL was analyzed it was observed that proteins account for 43% and lipids 35% of the storage matter dry weight. The major proteins have been shown to be saposins A and D, the two proteins existing in equal amounts (Tyynelä et al., 1993). Saposins are lysosomal proteins which are processed from the 70 kDa glycoprotein prosaposin to mature saposins A, B, C and D. These sphingolipid activator proteins are needed for degradation of glycosphingolipids (Furst and Sandhoff, 1992). Saposins are also accumulated in other lysosomal storage disorders, but in smaller amounts than in INCL (O'Brien and Kishimoto, 1991).

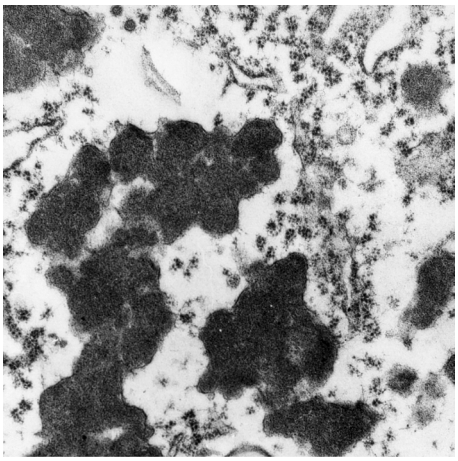

Figure 1. Granular osmiophilic deposits (GRODs) in the cortex of the INCL patient (autopsy sample). Magnification x18.000. (Courtesy of Prof. Juhani Rapola).

Storage material with curvilinear profiles (CL) is typical of classical late infantile NCL cases. These CL profiles are short, thin, lamellar stacks (dimension between 3-4 nm), which are membrane bound and usually fill the whole storage lysosome. Fingerprint (FP) bodies represent parallel paired dark lines of 7-9 nm with an internal thin clear space of 2 nm and these are typical of juvenile NCL. Rectilinear profiles (RL) often represent a pentalamellar stack (but several RL variants have also been described). RL profiles are part of various patterns found mainly in variant late infantile NCL forms (Carpenter et al., 1977).

In NCL, in contrast to the classical lysosomal storage disorders, biochemical analysis of the storage material did not uncover the underlying biochemical defects. Subunit c

of mitochondrial ATP synthase is a major protein accumulating in NCL subtypes other than INCL (Hall et al., 1991). In all cases, the storage lysosomes are present in several cell types of the nervous systems, but the lipopigments are also present in large numbers of extraneural cells. The storage material is detectable even in the placenta and the amniotic fluid cells of affected fetuses (MacLeod et al., 1988; Rapola et al., 1990; Munroe et al., 1996; Rapola et al., 1999). Variant forms of NCL show a mixed profile with more than one ultrastructure. The diagnosis of NCL subtypes has traditionally been based on clinical features and electron microscopic analysis, but nowadays DNA and biochemical tests dominate. For those families in which the gene defect is already known, it is possible to screen the mutations by sequencing and offer prenatal diagnostics when needed. The enzyme activity assays are available for INCL and classical LINCL (van Diggelen et al., 1999). However, investigation with electron microscopy is still needed when the causative gene defect is unknown.

1.5 Infantile types of neuronal ceroid lipofuscinosis

1.5.1 Infantile neuronal ceroid lipofuscinosis (CLN1)

INCL is the earliest and the most severe form of those NCL disorders in which the molecular background is known. Development in INCL is normal until the age of 6 to 18 months. Most of the children learn to say a few words and walk unaided before progressive deterioration of their skills starts. The first signs of the disease are retardation of psychomotor development and a decreased rate of head growth. At the age of 8 months, muscular hypotonia and progressive microcephaly are detectable and are soon followed by severe mental retardation, blindness, and epilepsy. By the age of 3 years almost all cortical functions are lost and the EEG of patients is isoelectric. Death occurs between 6 and 15 years of age (Santavuori et al., 1973; Santavuori et al., 1974). At autopsy, the brains are extremely atrophic, weighing 250 to 450 g (normally 1000 g in age-matched controls). The cerebral cortex has almost totally disappeared and can hardly be distinguished. Instead, large numbers of glial cells filled with accumulating material are clustered (Haltia et al., 1973a). The cerebellum is also atrophic, but the brain stem and spinal cord have an almost normal appearance (Fig. 2). The optic nerves are also severely atrophic: the myelin sheaths have vanished and are replaced by glial tissues (Tarkkanen et al., 1977).

The molecular basis of INCL was clarified by positional cloning of the CLN1 gene (Järvelä et al., 1991; Vesa et al., 1995). All cases of INCL have mutations in the palmitoyl protein thioesterase (PPT1) gene. Today 37 disease causing mutations have been characterized in PPT1 gene (Vesa et al., 1995; Das et al., 1998; Mitchison et al., 1998; Munroe et al., 1998; Santorelli et al., 1998; I; Waliany et al., 2000). PPT1 modifies several proteins *in vitro*, by removing fatty acyl groups from S-acylated proteins such as oncogene H-ras and α subunits of heterotrimeric G proteins (Camp and Hofmann, 1993). The mature PPT1 enzyme is a doublet of 39/37 kDa. PPT1 expressed in COS-1 cells is targeted to lysosomes through the mannose-6-phosphate

receptor pathway (Hellsten et al., 1996; Verkruyse and Hofmann, 1996). The molecular genetics and cell biology of PPT1 is reviewed more thoroughly below in part 3.3.1.

INCL brain

Control brain

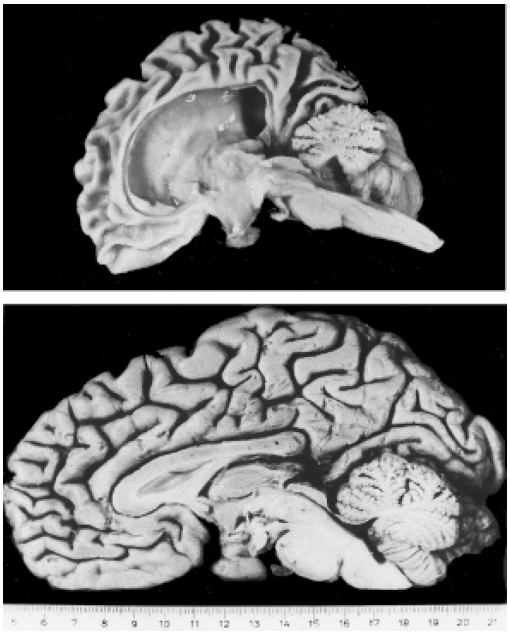

Figure 2. The INCL brain compared with a normal, age-matched control brain (Courtesy of Prof. Juhani Rapola).

1.5.2 Congenital neuronal ceroid lipofuscinosis

Congenital neuronal ceroid lipofuscinosis was initially described by Norman and Wood in 1941 (Norman and Wood, 1941). The disease is rare and rapidly fatal, death occurs between 24 h and 7 weeks after birth. The cerebellum is atrophic and massive astrocytosis is seen in the cerebral cortex. The neuropathology is almost identical to that of INCL, but the age of onset is notably earlier (Humphreys et al., 1985). The ultrastructure of the storage material is GROD (Garborg et al., 1987; Kolschutter and Lake, 1999). Neither the genetic loci nor the biochemical defect for congenital NCL is yet known.

1.6 Late infantile types of neuronal ceroid lipofuscinosis

LINCL is a diverse group and several variant forms have been described. At the moment, the chromosomal location is known for four variant types of LINCL and two genes have been characterized, but at least two genes have still to be identified.

1.6.1 Classical late infantile neuronal ceroid lipofuscinosis (CLN2)

The first symptoms of LINCL can be observed at the age of 2-4 years (Kohlschutter et al., 1993; Williams et al., 1999a). These include epilepsy, seizures, myoclonus and ataxia which are followed by gradual visual failure, leading to blindness at the age of 5 or 6 years. In the cortex, a remarkable decrease of neurons is observed (Gonatas et al., 1968). The gene is located on chromosome 11p15 (Sharp et al., 1997) and the defective gene responsible for classical LINCL is a lysosomal protease with tripeptidyl-peptidase I activity (TPP1) (Sleat et al., 1997; Rawlings and Barrett, 1999; Vines and Warburton, 1999). TPP1 is expressed ubiquitously in all tissues and its function is to remove N-terminal tripeptides from substrates having free amino termini. TPP1 encodes a 536 aa protein, which is predicted to contain a 19 aa signal sequence. The enzyme is synthesized as a 67 kDa precursor protein, which is processed to a 46 kDa mature protein (Liu et al., 1998). TPP1 is synthesized as an inactive proenzyme which, in acid pH, is converted into an active serine protease (Lin et al., 2001). *In vitro*, this enzyme has a role in the lysosomal degradation of subunit c of the mitochondrial ATP synthase (Ezaki et al., 1999). *In vivo*, the substrate(s) is not currently known. Forty different mutations have been reported in the TPP1 gene (Sleat et al., 1997; Caillaud et al., 1999; Hartikainen et al., 1999; Sleat et al., 1999; Berry-Kravis et al., 2000; Zhong et al., 2000; Lam et al., 2001).

1.6.2 Variant late infantile neuronal ceroid lipofuscinosis (CLN5), Finnish type

The Finnish variant of LINCL ($vLINCL_{Fin}$) was described by Santavuori and coworkers from a cluster of 16 families in Southern Ostrobothnia in Finland (Santavuori et al., 1982). The disease begins between 4 and 7 years of age and the first symptom is motor clumsiness. Other symptoms include progressive visual failure, mental retardation, and motor deterioration which are followed by myoclonia and seizures. Death occurs between 13 and 30 years. At autopsy, the most striking abnormality in the brain is the cerebellar atrophy. The ultrastructure of the storage material shows curvilinear bodies and fingerprint profiles (Santavuori, 1988; Tyynelä et al., 1997b). The CLN5 gene is located on chromosome 13q22 (Klockars et al., 1996). The causative gene in $vLINCL_{Fin}$ consists of four exons spanning 13 kb of genomic DNA. The coding region of the cDNA has a 1380 bp open reading frame and the estimated size of the CLN5 protein is 46 kDa, with two hydrophobic regions potentially representing transmembrane domains (Savukoski et al., 1998). Very recent data indicate that the CLN5 polypeptide is targeted to the lysosomes in non-neuronal cell lines (J. Vesa and J. Isosomppi, personal communication). Four mutations

underlying $vLINCL_{Fin}$ have been characterized (Savukoski et al., 1998; Holmberg et al., 2000), but the actual disease mechanism still remains to be solved.

1.6.3 Variant late infantile neuronal ceroid lipofuscinosis (CLN6)

Clinically, vLINCL has a slightly later onset than the classical LINCL (age of onset 4-5 years) and the course of the disease is more protracted. The age at death is 10-30 years. Most of the patients are from Portugal, India, or Pakistan. In electron microscopy, mixed curvilinear and fingerprint inclusions are seen (Lake and Cavanagh, 1978; Williams et al., 1999b). The chromosomal location of the CLN6 gene is 15q21-23 (Sharp et al., 1997), but, so far, the gene has not been isolated.

1.6.4 Variant late infantile neuronal ceroid lipofuscinosis (CLN7), Turkish type

In the third variant form of LINCL, all the patients are of Turkish descent (Williams et al., 1999c). The first signs of the disease are seizures and poor mobility. Visual deterioration has a variable onset. These symptoms are followed by motor and cognitive deterioration. The CLN7 gene has recently been mapped to 8p23, and further experiments will clarify whether CLN7 is allelic with CLN8 (Mitchell et al., 2001). The gene defect underlying this disorder has still to be clarified.

1.7 Juvenile types of neuronal ceroid lipofuscinosis

1.7.1 Juvenile neuronal ceroid lipofuscinosis (CLN3)

Worldwide, JNCL represents the most common form of NCL. JNCL usually begins with visual failure at 5-10 years of age. Mental retardation develops slowly and is followed by epilepsy and deterioration of motor skills. Patients become nonambulant at 13-30 years and death occurs in the second or third decade. At autopsy, the cerebral cortex is narrowed and the weight of the brain is decreased. Electron microscopy reveals fingerprint profiles (Carpenter et al., 1977). Vacuolated lymphocytes at light microscopic level are a constant feature, which is unique among the NCL disorders (von Bagh and Hortling, 1948; Santavuori, 1988). The CLN3 gene is located at 16p12 (Gardiner et al., 1990) and collaboration of five research groups led to isolation of the CLN3 gene in 1995 (The International Batten Disease Consortium, 1995). The length of this gene is 15 kb and the mRNA has an open reading frame of 1314 bp. Computer-based predictions of the amino acid sequence of this 43 kDa protein with no homology to any other known protein indicates 5-7 hydrophobic regions, suggesting that it is an integral transmembrane protein (Mitchison et al., 1997). The intracellular localization of the CLN3 protein has been reported to be lysosomal in nonneuronal cells (Järvelä et al., 1998), but mitochondrial (Katz et al., 1997), Golgi compartment (Kremmidiotis et al., 1999) and nuclear or cytoplasmic (Margraf et al., 1999) localization have also been suggested. In mouse primary neurons, the CLN3 protein localizes predominantly in synaptosomes of the presynaptic nerve terminals (Luiro et al., 2001). The CLN3 protein has a yeast homolog, Btn1, and studies with the yeast Btn1 knock-out model

have suggested a possible role for Btn1 in the regulation of vacuolar pH or vesicular trafficking (Pearce et al., 1999a; Pearce et al., 1999b; Chattopadhyay et al., 2000). However, the function of the human CLN3 still remains elusive. At the moment, 31 different mutations in the CLN3 gene have been characterized, of which the most common one is a 1.02 kb deletion leading to skipping of exons 7 and 8 and early truncation of the protein (The et al., 1995; Mitchison et al., 1997; Munroe et al., 1997; Zhong et al., 1998).

1.7.2 Northern epilepsy with mental retardation (CLN8)

The most recent discovered form of NCLs is epilepsy with progressive mental retardation (EPMR). The locus for EPMR is CLN8. This childhood epilepsy with autosomal recessive inheritance starts with tonic-clonic seizures at 5 to 10 years of age. Slowly progressive mental deterioration is followed 2 to 5 years after the first seizures. The life expectancy of patients is 50 to 60 years. There is no visual failure in this disorder. Cerebellar atrophy is seen in all the patients, but neuronal death in the cortex is clearly less severe than in JNCL (Hirvasniemi et al., 1994). The storage material is curvilinear profiles, but granular material is also seen (Haltia et al., 1999). The gene is located in 8p23 (Tahvanainen et al., 1994; Ranta et al., 1997). The gene encodes a novel 286 aa transmembrane protein (Ranta et al., 1999). The CLN8 protein contains an ER retrieval signal and it has been shown to be located in the ER and also in the ER-Golgi intermediate compartment (Lonka et al., 2000). One mutation in the CLN8 gene has been characterized (Ranta et al., 1999).

1.8 Adult types of neuronal ceroid lipofuscinosis (CLN4)

1.8.1 Kuf's disease and Parry's disease

Adult neuronal ceroid lipofuscinosis (ANCL) is a rare heterogeneous disorder. Kuf's disease and Parry's disease are two reported forms of ANCLs. In these NCL disorders there is no visual failure. The leading symptom of the patients is dementia, followed by myoclonus. The age of onset varies between 10 and 55 years. Inheritance may be either autosomal recessive or autosomal dominant (Boehme et al., 1971; Berkovic et al., 1988). Electron microscopy has revealed a mixture of profiles including curvilinear, fingerprint, and GROD profiles (Ruchoux and Goebel, 1997). Neither the gene location nor the gene product for these diseases is known. All mutations known in the NCL genes are collected in an NCL mutation database (http://www.ucl.ac.uk/ncl).

Clinical onset	*NCL disorder*	*Ultra-Structure*	*Storage*	*Locus*	*Gene location*	*Gene product*	*MIM*	*Cases in Finland**
Infantile	Infantile	GROD	Saposins A+D	CLN1	1p32	Palmitoyl protein thioesterase1	256730	158
Late infantile	Classical Late infantile	CL	Mitoch. Subunit c	CLN2	11p15	Tripetidyl peptidase 1	204500	5
	Finnish variant	FP CL RL	Mitoch. Subunit c	CLN5	13q22	Lysosomal membrane protein	256731	26
	Variant	FP CL RL	Mitoch. Subunit c	CLN6	15q21-23	?	601780	
	Turkish variant	FP CL RL	Mitoch. Subunit c	CLN7	8p23	?	--	
Juvenile	Juvenile	FP (CL RL)	Mitoch. Subunit c	CLN3	16p12	Lysosomal membrane protein	304200	185
	Northern epilepsy	CL	Mitoch. Subunit c	CLN8	8p23	Transmembr. protein of the ER-ERGIC	600143	26
Adult	Kuf's Parry's	GROD FP	Mitoch. Subunit c	CLN4	?	?	204300 162350	1

Table 2. Classification of neuronal ceroid lipofuscinoses.
GROD = granular osmiophilic deposits, CL = curvilinear profiles, FP = fingerprint profiles, RL= rectilinear profiles, Mitoch. Subunit c = subunit c of the mitochondrial ATP synthase. *Santavuori et al., 2000.

1.9 Animal models

Naturally occurring animal models for NCL have been described in several animal species, including dog, cat, sheep, goat, cow, and mouse (Table 3). All the affected animals have brain atrophy. Moreover, the ultrastructure of the storage materials is either lamellar profiles, when the accumulating material is subunit c of the mitochondrial ATP synthase, or GROD structure with accumulating saposins A and D.

At least 15 dog breeds have been described to have an NCL disorder. The miniature Schnauzer is the only one in which the appearance of the storage material is GROD and the accumulating materials are saposins A and D (Palmer et al., 1997b). The first sign of the disease is progressive blindness at the age of 3-4 years. The other example of late onset of clinical symptoms is the Tibetan terrier. Progressive clumsiness and ataxia is observed at the age of 4 years, followed by severe visual failure (Alroy et al., 1992).

The clinical signs are quite like those of human adult CLN4 disorder, except that no visual problem occurs in human CLN4. In the case of the English setter (Koppang, 1992) and the Border collie (Palmer et al., 1997a), the first clinical symptoms are progressive incoordination and loss of vision arising around 1 year of age. The human equivalent for these disorders, is currently not known although both species have been studied extensively. The disease of the English setter is not linked to the syntenic region of human the CLN3 or the CLN2 gene (Lingaas et al., 1998).

The Swedish landrace sheep is a congenital ovine NCL (CONCL) model. The appearance of the storage material is GROD and the accumulating substances are saposins A and D (Tyynelä et al., 1997a). The newborn lambs are weak, trembling, and unable to rise. When bottle fed, affected animals survive for a few weeks (Järplid and Haltia, 1993). Earlier, it was assumed that these animals represented models for human INCL, but their PPT1 activity is normal. More recent investigations have revealed that the defect in CONCL is mutation in the cathepsin D gene, which is a lysosomal aspartyl proteinase (Tyynelä et al., 2000). It will be interesting to find out whether the human equivalent of this disorder is congenital NCL, which is also a rapidly fatal NCL disorder. In South Hampshire sheep, the age of onset is 1 year and the average life span of affected animals is two years. Impaired vision and progressive brain atrophy are typical (Jolly et al., 1992). The disease in South Hampshire sheep (Broom et al., 1998) and in Australian merino sheep are probably equivalent to the human variant LINCL, CLN6 because the disease gene in each case has been localized to conserved synteny with the region on human chromosome 15q (Tammen et al., 2001).

Two naturally occurring mouse models for NCL disorders have been characterized. The motor neuron degeneration (mnd) mouse is characterized by early progressive retinal atrophy followed by motor dysfunction and premature death at 12 months of age (Bronson et al., 1993; Messer et al., 1993). The mnd mouse has a mutation in the CLN8 gene (Ranta et al., 1999). Neuronal ceroid lipofuscinosis (nclf) mice also have progressive retinal atrophy and motoneuronal degeneration. The progression of the disease is slower than in the mnd mouse. Nclf is a homolog of human vLINCL, CLN6 (Bronson et al., 1998). Three knock-out mouse models for CLN3 have been published (Greene et al., 1999; Katz et al., 1999; Mitchison et al., 1999). These mice have accumulations of characteristic storage material in the neurons. At the age of one year, homozygous CLN3 mice are viable and fertile, with no obvious symptoms (Greene et al., 2001).

In conclusion, currently no naturally occurring animal models for CLN1, CLN2, CLN3, or CLN5 are known.

Animal	Age of onset	Storage protein	Human equivalent	Reference
Dog				
Miniature Schnauzer	Juvenile	Saposins A+D	Adult?	Palmer et al., 1997b; Tyynelä et al., 1997a
Tibetan Terrier	Juvenile	Mitoch. Subunit c	Adult?	Riis et al., 1992; Palmer et al., 1997a
English setter	Juvenile	Mitoch. Subunit c	?	Koppang, 1992; Palmer et al., 1997a
Border collie	Juvenile	Mitoch. Subunit c	?	Palmer et al., 1997a
Sheep				
Swedish Landrace	Infantile	Saposins A+D	Congen (?)	Järplid and Haltia, 1993
South Hampshire	Juvenile	Mitoch. Subunit c	CLN6	Jolly et al., 1992; Broom et al., 1998
Australian Merino	Juvenile	Mitoch. Subunit c	CLN6 (?)	Tammen et al., 2001
Mice				
Mnd	Juvenile	Mitoch. Subunit c	CLN8	Bronson et al., 1993; Ranta et al., 1999
Nclf	Juvenile	Mitoch. Subunit c	CLN6	Bronson et al., 1998
Knock-out CLN3	Juvenile	Mitoch. Subunit c	CLN3	Greene et al., 1999; Katz et al., 1999; Mitchison et al., 1999

Table 3. Animal models for neuronal ceroid lipofuscinosis.

The pathological cascade from a gene defect to accumulating material and eventually to degeneration of nerve cells has remained elusive. Animal models offer tools for studying the biochemical mechanisms underlying these disorders. At the moment, treatment of all NCL disorders is supportive. Bone marrow transplantation (Lake et al., 1997), dietary attempts (Bennett et al., 1994) and stem cell transplantation (Lönnqvist et al., 2001) have been tested, but currently there is no curative treatment for NCL. Specific therapy of the central nervous system is extremely difficult. Animal models are very valuable for testing and developing future therapies. The main challenges are how to cross the blood-brain barrier and how to stop the neuronal degradation.

2. Transport of proteins after synthesis

In general, when the pathogenesis of a disease is clarified, we first need to know how the proteins are localized. Here the protein trafficking of the secretory pathway is reviewed, since current knowledge indicates that all NCL proteins are targeted to this pathway.

2.1 Transport of proteins through the ER and the Golgi complex

Soluble proteins destined for translocation into endoplasmic reticulum (ER) have a hydrophobic signal sequence at their N-terminus. For transmembrane proteins this translocation signal may also be internal, being the first transmembrane region. Soluble proteins are translocated inside the ER lumen and transmembrane proteins are inserted into the ER membrane (Wilkinson et al., 1997; Kalies and Hartmann, 1998). Membrane-bound ribosomes synthesize proteins of three main classes: 1) secretory proteins, 2) proteins spanning the plasma membrane, and 3) proteins destined for various compartments of the secretory system. After translocation, chaperones (ER-resident proteins) in the lumen of the ER bind to newly synthesized proteins and maintain them in a state suitable for subsequent folding (Hartl, 1996; Bukau and Horwich, 1998). In the ER lumen the proteins are modified: Amino terminal signal sequence is cleaved immediately after synthesis, disulfide bridges are formed, and N-linked glycosylation (addition of an oligosaccharide group to the NH_2 group of asparagine) is initiated (Kornfeld and Kornfeld, 1985; Helenius and Aebi, 2001). Three major types of lipid modification have been identified: 1) myristoylation, which is a cotranslational process occurring in the ER, and 2) prenylation, and 3) palmitoylation, which occur posttranslationally in the ER or in the cis-Golgi compartment (Morello and Bouvier, 1996). ER-resident soluble proteins have a short sequence at the C terminus (KDEL in single aa letter code) (Munro and Pelham, 1987; Pelham, 1990). If this sequence is deleted, the protein is secreted instead of remaining in the lumen of the ER. The signal responsible for ER membrane localization contains two lysine residues (KKXX) located in the cytoplasmic tail prior to the C terminus (Nilsson et al., 1989; Jackson et al., 1990; Brown and Breton, 2000).

Transport of material from the ER to the trans-Golgi complex is involved through the ER-Golgi intermediate compartment (ERGIC) (Saraste and Kuismanen, 1984). The structures restricting ER, ERGIC, and the Golgi complex are not clearly defined. ERGIC plays a role as a sorting element between anterograde (ER to Golgi) and retrograde (Golgi to ER) transport. Export from the ER to the ERGIC complex occurs in COPII coated vesicles (Barlowe et al., 1994). Currently, the COPII coated vesicles are not fully characterized, but they are known to contain at least the coatomer protein and GTPase (Antonny and Schekman, 2001). Transport from the ERGIC to the cis-Golgi compartment and through the Golgi complex occurs in COPI coated vesicles (Serafini et al., 1991b). The retrograde transport from the Golgi complex to the ER is also mediated by COPI coated vesicles. The components of the COPI vesicles are ARF (ADP ribosylation factor, GTPase) (Serafini et al., 1991a), and the coatomer protein

complex, consisting of seven subunits of the stable cytosolic protein complex (Waters et al., 1991; Barlowe, 2000). Both COP-coated vesicles operate mainly in transport through the early secretory pathways and, on the basis of their coats, COPI and COPII are capable of carrying out appropriate targeting.

Correctly folded proteins are transported to the Golgi complex, while incorrectly folded proteins are retained in the ER. The Golgi complex is the major sorting center of the cell, targeting proteins to their proper locations. Both secretory and membrane proteins are carried to the Golgi complex by small transport vesicles. The Golgi complex is polarized, having two distinct membranes. Proteins exported from the ER enter the cis-Golgi compartment and continue their passage through the several compartments of the Golgi complex. Glycoproteins are modified in each compartment of the Golgi complex. N-linked glycosylation, which was initiated in the ER, is completed in the Golgi compartment (Kornfeld and Kornfeld, 1985), while O-linked glycosylation (addition of an oligosaccharide group to the OH-group of serine, threonine or hydroxylysine) occurs in the Golgi complex alone (Wells et al., 2001). The trans-Golgi network, facing the plasma membrane, is the exit site from the Golgi complex where proteins are directed to their destinations. Despite the continuous flux of proteins, a certain set of proteins must be Golgi-resident. For membrane proteins, the cytoplasmic domain of about 100 residues adjacent to the COOH-terminal membrane-anchoring domain is responsible for Golgi localization (Misumi et al., 2001). Transport from the trans-Golgi network to the target organelle or to the plasma membrane is mediated either by COPI coated or clathrin-AP1 coated vesicles. The clathrin coated vesicles are coated with two layers: the outer layer consists of clathrin, while the inner layer is formed by an adaptor protein (AP), which binds to clathrin and also to the integral membrane proteins of the vesicle (Keen, 1987; Hirst and Robinson, 1998).

2.2 Transport of proteins to lysosomes

Lysosomes are acidic membrane-bound organelles that are rich in hydrolytic enzymes and are responsible for controlled intracellular digestion of macromolecules (Kornfeld and Mellman, 1989). Transport of lysosomal hydrolases to the lysosome is the best-characterized example of diverse sorting processes mediated by different transport vesicles. The marker for lysosomal transport is mannose-6-phosphate (M6P) (Kaplan et al., 1977). M6P groups are added to the N-linked oligosaccharides in the cis-Golgi network. This is mediated by a three-dimensional binding signal for the N-acetylglucosamine phosphotransferase (von Figura and Hasilik, 1986). M6P receptors (MPR46 and MPR300) are transmembrane proteins present in the trans-Golgi network (Kornfeld and Kornfeld, 1985; Kornfeld, 1992; Hille-Rehfeld, 1995). These receptors bind to the lysosomal hydrolases marked by M6P in the neutral pH of the Golgi and help to package them into specific vesicles. Vesicles containing this receptor protein complex bud off from the trans Golgi compartment and fuse with the early endosome. The lower pH of the endosome causes dissociation of the protein from the receptor, which is recycled back to the Golgi compartment for new rounds of transport (Duncan and Kornfeld, 1988). Other targeting mechanisms not depending on M6P have also been characterized. Most of the lysosomal membrane proteins are delivered to the

lysosomes by tyrosine-based (Chen et al., 1990) and/or dileucine-based (Corvera et al., 1994) sorting signals, which are present in the cytoplasmic domains of the proteins. These sequences are recognized by clathrin-coated vesicles containing the appropriate adaptor protein (Brown and Breton, 2000).

Other receptors besides M6PR have been reported to be responsible for lysosomal targeting of soluble proteins. An alternative, but partly M6P receptor-dependent, trafficking is reported for endocytosis of the saposin precursor. From the ER, saposin precursors are transported either along the secretory pathway to the lysosome or, alternatively, this precursor is secreted from the cell. Intracellular transport is mediated by the M6P receptor. The secreted saposin precursors are endocytosed back in to the cell by receptor-mediated endocytosis (Vielhaber et al., 1996). This is performed mainly by the LRP (low density lipoprotein receptor-related protein) and to a lesser extent with the M6P receptor. LRP is a endocytic cell surface receptor, which is expressed ubiquitously. LRP also functions in the cellular uptake of, for example, proteases and protease inhibitor complexes (Hiesberger et al., 1998).

2.3 The endosomal-lysosomal pathway

The endocytic pathway is responsible for the internalization of extracellular material known as endocytosis. Exocytosis is the converse process, in which, intracellular material is transported to the extracellular space. The exocytic and endocytic pathways communicate through the exchange of materials between the Golgi complex and the endosomal elements (Mellman, 1996). Early endosomes are located near the plasma membrane and are the main sorting station of the endocytic pathway (Mukherjee et al., 1997). The interior of the endosomal compartment is acid (pH 6). Material can be recycled back to the plasma membrane, some molecules are destined to be degraded and transported to lysosomes, and are therefore first transported to the late endosomes, which are located near the Golgi complex (Mellman, 1996). A pathway from the early endosomes to the trans-Golgi network has been reported (Mallard et al., 1998), as well as from the late endosomes to the trans-Golgi network (Kornfeld, 1992). It is thought that hydrolytic digestion of the endocytosed molecules begins in the late endosomes. The material from the late endosomes is transported to the lysosomes (Kornfeld and Mellman, 1989). The relationships between the early and late endosomes and also the lysosomes are not clear. One view (the maturation model) is that endosomes are formed when transported molecules fuse. On fusion of incoming material, the early endosomal compartment matures to the late endosome (Thilo et al., 1995). Another view (stationary organelles) is that the early and late endosomes are stable compartments and that transport between them occurs via the endosomal carrier vesicles (Aniento et al., 1993). There is no evidence in support of vesicular transport between the late endosomes and the lysosomes. Recent data suggests that lysosomes fuse directly with late endosomes, forming a hybrid organelle, in which the endocytosed material is degraded. According to this model, lysosomes are re-formed from the resulting organelle (Luzio et al., 2000) (Fig. 3).

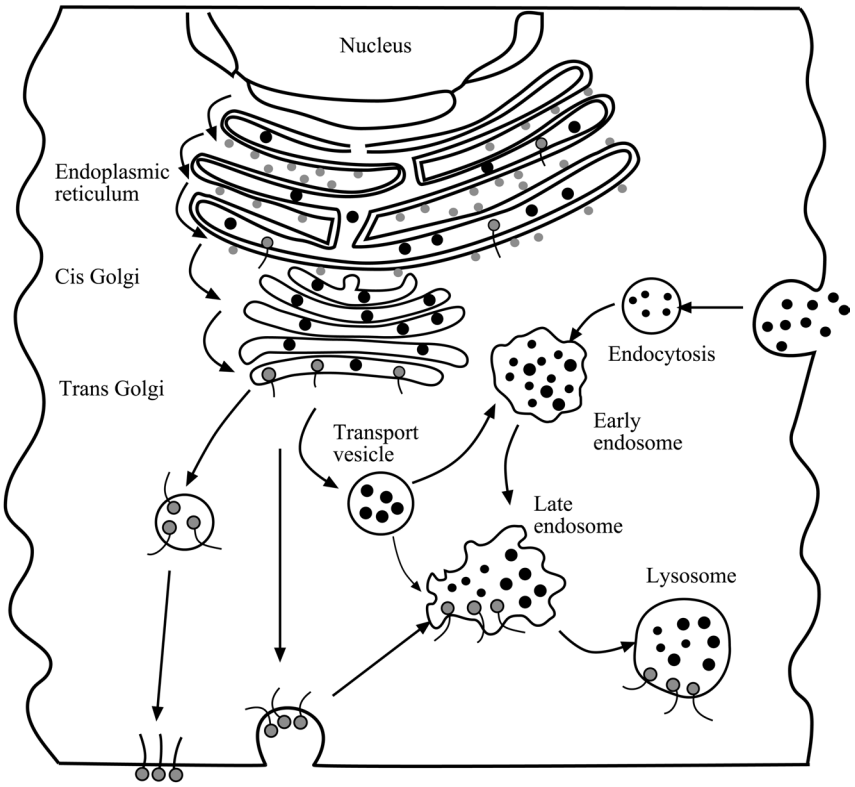

Figure 3. Transport of the newly synthesized proteins along the secretory pathway. After synthesis by ER-bound ribosomes, proteins are translocated in the ER. From the ER, proteins are transported to the Golgi complex. The trans-Golgi network is a sorting center directing proteins to different locations, for example, to the plasma membrane or to the endosome.

Briefly, the steps in the vesicle-mediated transport of material consist of generation of coated vesicles containing the endocytosed material, pinching off of the vesicle, uncoating of the protein layer, reaction between the protein in the vesicle membrane and the receptor in the target membrane, followed by fusion with the target membrane and release of the transported molecules. Cytosolic coat components bind to the membrane of the donor compartment, deforming it, and a coated vesicle is generated. This membrane vesicle is pinched off and transported to the early endosome. Protein transport from the plasma membrane to the early endosomes is mediated by small and short-lived vesicles coated with clathrin and AP2 (Robinson, 1994). Transport from the early to the late endosomes is carried out by relatively large and long-lived COPI coated endosomal carrier vesicles (Kreis et al., 1995).

Budding of clathrin-coated vesicles is initiated by the GTP-binding protein ARF (ADP-ribosylation factor). ARF is N-myristoylated, allowing its insertion into the membrane. Membrane-bound active ARF-GTP recruits subunits of the coating protein into the membrane, causing budding of a vesicle (Donaldson et al., 1992). Fusion of the vesicle

with the target membrane demands uncoating. Hydrolysis of the ARF-GTP results in dissociation of the coat (Tanigawa et al., 1993). The specificity of the docking and fusion of the vesicle to the proper target membrane is provided by the SNARE (SNAP receptor) complex (Rothman, 1994; Rothman and Wieland, 1996) (Fig. 4). Originally SNARE complex was characterized as a requirement for fusion between synaptic vesicle and plasma membrane in nerve cells. This led to the search for non-neuronal homologies of this fusion system and isoforms of the neuronal SNARE proteins were identified, for example, in fat and muscle cells (Ravichandran et al., 1996). The components belonging to this complex are NSF (identified by its sensitivity to the sulfhydryl agent N-ethyl-maleimide) which acts as a soluble ATPase (Block et al., 1988), SNAP (a soluble NSF attachment protein, binds to the membrane) (Clary et al., 1990) and the SNARE (the SNAP receptor) (Sollner et al., 1993). The hydrolysis of ATP dissociates the NSF-SNAP complex, allowing the recycling of these fusion particles. SNARE molecules have distinct subcellular distributions: vSNARE represents SNARE associated with the donor vesicles and tSNARE is associated with the target. The specificity of vesicle targeting is guaranteed partly by a family of GTP-binding proteins called rab which controll that the fit between vSNARE and tSNARE is correct. Over 30 different rab proteins are known and each type is associated with a particular membrane-bound organelle (Somsel Rodman and Wandinger-Ness, 2000).

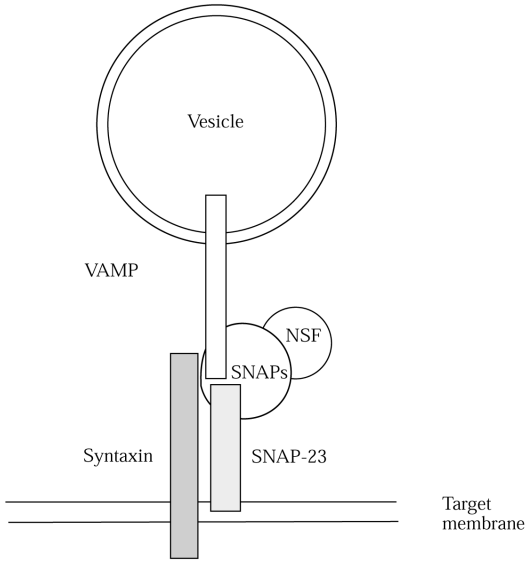

Figure 4. The role of SNAREs in vesicular transport. v-SNARE (vesicle SNARE) consists of the membrane protein VAMP (vesicle association membrane protein). t-SNARE (target membrane SNARE) is consists of the transmembrane protein syntaxin and SNAP-23, which is connected to the membrane via acylation. The v-SNARE recognizes the t-SNARE, forming a docking complex to which SNAP and NSF bind.

2.4 Specific features of protein trafficking in neurons

Nerve cells are specialized for transforming information. The strictly regulated secretion of neurotransmitters in synapses of the nervous system is a premature form of communication. This system has certain unique features: Exocytosis is calcium-regulated and the reaction occurs at high speed (Augustine et al., 1987). Certain classes of proteins are involved in the intracellular fusion reactions in neuronal and non-neuronal cells. For example, SNARE proteins, rab proteins, and the clathrin coat are universal.

The machinery required for protein trafficking is highly conserved. Studies of the secretory pathway in the yeast S. cerevisiae carrying different secretory mutations and the secretory machinery of vertebrate synapses have shown that the structural components of the vesicles and proteins participating in budding and in attachment of the vesicles are universal (Pevsner, 1996). Polarized epithelial cells (having two different membranes) have been used as a model for studying polarity and the targeting of proteins to the neuronal plasma membrane. A typical epithelial cell has an apical domain, which has specialized features, and a basolateral domain covering the rest of the cell (Matlin and Simons, 1984). The original proposal was that the apical membrane of the epithelial cells was equivalent to the axonal sites and the basolateral membranes were equivalent to the nerve cell body and the dendrites (Dotti and Simons, 1990). Later on, however, it has been demonstrated that this is not always the case (Jareb and Banker, 1998). The protein and lipid compositions of these two membranes are different and they are demarcated by tight junctions (Pereira et al., 1995). The sorting center for apical and basolateral targeting is the trans-Golgi compartment (Keller and Simons, 1997). Different types of secretory vesicles are targeted to different types of membranes and certain targeting signals for proteins have been characterized. A tyrosine-based signal directs the basolateral targeting of proteins, but many basolateral proteins are lacking this signal. Apical targeting signals need more characterization (Brown and Breton, 2000). Endocytosed material can be transported across the cell to membranes with distinct polarity (from the apical to the basolateral membrane or vice versa). This is called transcytosis (Fabian, 1991).

Neurons are an extreme example of polarization, having distinctive regions: the cell body contains the nucleus and most of the ribosomes, the endoplasmic reticulum, the Golgi complex and the lysosomes. Multiple dendrites are specialized to receive information and usually one axon conducts the information away from the cell body. Intracellular transport is extremely active in neurons. Most of the axonal proteins are synthesized in the cell soma. These proteins, as well as the membrane-bound organelles budding off the trans-Golgi network in the cell body, must be transported down the length of the axon to the synaptic end (Jahn and Südhof, 1993) (Fig. 5). There are two distinct routes for axonal transport. Slow actin-based transport of cytoplasmic proteins (Pfister, 1999) and fast transport of membrane-bound organelles. Fast transport is based on movements of organelles along microtubules. Kinesin is a fast axonal transport motor, which is competent for binding a transport organelle (Martin et al., 1999). APP (amyloid precursor protein) is an example of neuronal transcytosis. APP is synthesized

in the cell soma and transported to the axon and is subsequently found in the dendrites (Simons et al., 1995). Some axonal proteins are synthesized locally. The machinery is localized in the cortical zone of the axon. These specific mRNAs and ribosomes synthesize proteins, most of which appear to be constituents of the slow axonal transport group (Koenig and Giuditta, 1999).

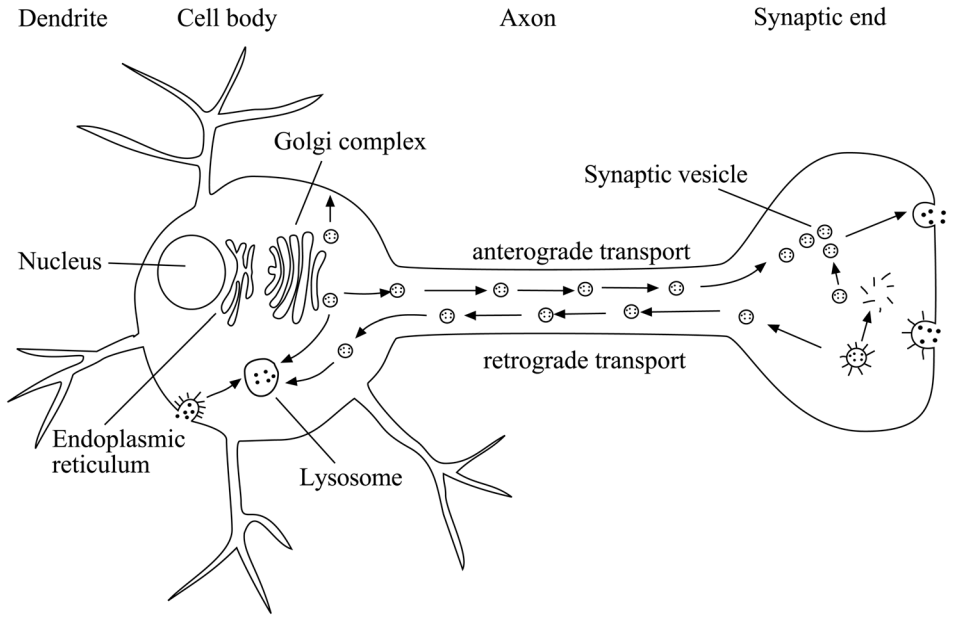

Figure 5. Schematic presentation of a nerve cell, showing transport of proteins and formation of synaptic vesicles. Modified from Neurobiology, Gordon M. Shepard, edit. (1988), pp.58, Oxford university press.

During recent years, studies of the synaptic vesicles in nerve terminals have been intensive. A large number of presynaptic proteins have been identified and protein-protein interactions have been shown (Südhof, 2000). Basically, the secretion of neurotransmitters consists of filling the synaptic vesicles with the proper neurotransmitters, transport to the presynaptic plasma membrane, docking to membrane, exocytosis, and recycling of the membranes (Fig. 6). Synaptic membranes are synthesized in the trans-Golgi network in the cell body (De Camilli and Jahn, 1990) and their size is smaller than that of the secretory vesicles in other cells. It has been proposed that the synaptic vesicles comprise three pools: a resting pool, a reserve pool and a readily releasable pool which contains docked vesicles that are immediately available for release (Murthy and Stevens, 1999). Membrane components of the synaptic vesicles are delivered to the plasma membrane by the constitutive secretory pathway, retrieved inside the cell by endocytosis, and transported to the endosomes, where they are reassembled, followed by budding of the synaptic vesicles. In a shorter

pathway, the synaptic vesicles recycle directly without endosomal intermediates (Murthy and Stevens, 1998).

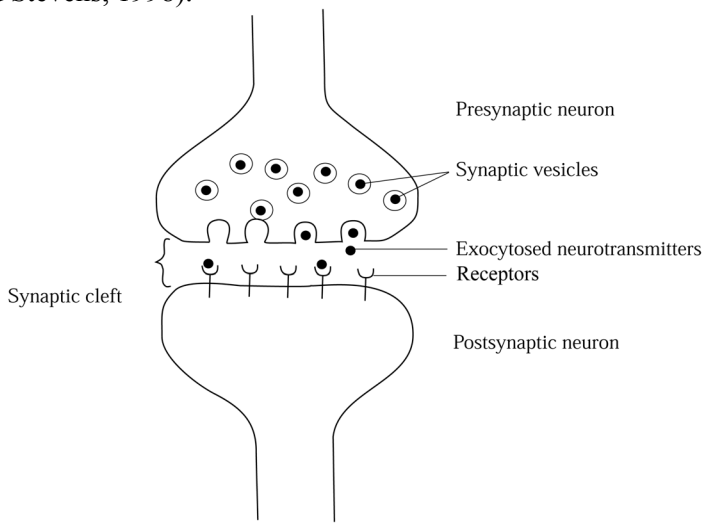

Figure 6. General structure of the synapse.
An impulse causes exocytosis and neurotransmitters stored in synaptic vesicles are released into the synaptic cleft.

2.4.1 Proteins involved in synaptic vesicle trafficking

Synapsins are a multigene family of phosphoproteins peripherally associated with the cytoplasmic surface of the synaptic vesicle membrane (Huttner et al., 1983). Synapsins regulate synaptic transmission and are involved in maintaining the synaptic vesicles in the reserve pool. The functioning of synapsins is dependent on Ca^{2+}. Mobilization of the synaptic vesicles from the reserve pool is probably regulated by phosphorylation of synapsins by calcium-regulated kinases (Llinas et al., 1991; Greengard et al., 1993). The mobilization of vesicles to the presynaptic plasma membrane is followed by their attachment to this membrane, a process called docking. In vesicle fusion to the acceptor membrane, rab proteins are implicated. Rab3A is a synaptic vesicle associated rab protein, which has GTPase activity and GTP/GDP binding sites (Balch, 1990). Docking is followed by a priming reaction. Priming is an ATP-dependent reaction which prepares vesicles for fusion. For example, ATPase NSF dissociates SNARE proteins. The last step in membrane fusion is performed by the SNARE complex. The three synaptic membrane proteins originally characterized in the SNARE complex are synaptobrevin (also called VAMP-vesicle associated membrane protein) (Trimble et al., 1988; Baumert et al., 1989), syntaxin (Bennett et al., 1992), and SNAP-25 (synaptosome associated protein) (Oyler et al., 1989). The SNARE complex interacts with several proteins, for example, with the Ca^{2+} channels. Synaptotagmins are a family of calcium-binding proteins located in the integral part of the synaptic vesicle membrane (Li et al., 1995). These proteins are not found in yeast, suggesting that they

are not a part of the basic membrane-trafficking machinery. Synaptotagmins contain several domains, allowing intermolecular interactions. They have a calcium-binding domain and a region that interacts with the SNARE complex. Synaptotagmins play role in the fusion of vesicles to target membranes (Chapman et al., 1998). After fusion, the synaptic vesicle membrane is recycled back into the cell via endocytosis. This is performed by clathrin-mediated budding. However, the synaptic vesicles are not clathrin-coated and the endocytic vesicles probably give rise to the synaptic vesicles by breaking up their clathrin coats (Jahn and Südhof, 1993).

In conclusion: The trafficking machinery acting in synaptic vesicle transport has many similarities with membrane trafficking of all cells. The special demands on neuronal cells (high speed, Ca^{2+}-dependent regulation, and plasticity in transmitter release) have resulted in selection of synapse-specific proteins to act in conserved trafficking events. Particular proteins are needed to cope with special requirements (for example synaptobrevins) and are specialized, acting only in synapses. The understanding of the molecular trafficking events in presynaptic terminals requires a great deal of further clarification.

3. Lipid modifications of proteins

During and after translation, proteins are modified at specific locations by moieties of different kinds. Glycosylation and phosphorylation have been studied for decades, but in recent years increasing numbers of proteins have been found to be modified by lipids. These modifications can significantly change the conformation, location, and function of the proteins. The lipid moieties most commonly found are fatty acids and isoprenoids. Three major types of attachment of lipids to proteins are prenylation, myristoylation and palmitoylation (Table 4).

3.1 Prenylation and myristoylation

Prenylation (also called isoprenylation) is a covalent modification of proteins, which occurs by adding the isoprenoid derivative of mevalonic acid to the synthesized polypeptide. In prenylated proteins, the attached long-chain prenyl groups are farnesyl (C 15 isoprenoid) or geranylgeranyl (C 20 isoprenoid). About 0.5-2 % of mammalian proteins are prenylated and they participate in signal transduction pathways (Casey et al., 1989; Epstein et al., 1991). Prenylation is a post-translational modification and the CAAX consensus site for prenylation is located at the carboxy terminus of proteins. All known prenylated proteins are found to bind to cellular membranes (Clarke, 1992; Schafer and Rine, 1992). For example, rab proteins have several prenylation motifs at their C terminus. For activity, rab proteins must associate with cellular membranes and this is mediated by the geranygeranyl moiety (Desnoyers et al., 1996).

Myristoylated proteins are modified with the 14-carbon saturated fatty acid named myristate. Myristoylation occurs cotranslationally before the first 100 amino acids of

the nascent polypeptide chain are synthesized (Deichaite et al., 1988). Proteins are myristoylated when the initiator methionine is followed by the amino acid glycine. This methionine is removed by methionine aminopeptidase. Myristoyl-CoA is a fatty acid donor and the enzyme N-myristoyltransferase (NMT) adds the myristate to the N-terminal glycine residues. The consensus sequence for NMT is Met-Gly-X-X-X-Ser/Thr (Towler et al., 1987). Myristate represents less than 1% of the total fatty acids in cells (Johnson et al., 1994). The amide linkage formed between glycine and myristate is very stable and it has been suggested that myristoylation in a irreversible modification of proteins.

3.2 Palmitoylation

Palmitoylation is a post-translational event that refers to addition of the 16 carbon-saturated fatty acid palmitate to the side-chain of cysteine. Palmitoylation is also referred to as S-acylation, since it can be substituted by other fatty acids. In addition to palmitate (C16), stearate (C18), oleate (C18), and arachidonate (C20) have also been found to be incorporated into palmitoylation sites (Casey, 1995). The significance of these different fatty acid attachments to protein function is not known. The thioester bond formed by this interaction is labile, making removal of palmitate possible (Magee and Courtneidge, 1985; McIlhinney et al., 1985; Olson et al., 1985). Accordingly, controlled addition and removal of this moiety allows dynamic regulation of protein function. For example, G-proteins have been shown to be dynamically regulated by palmitoylation (O'Dowd et al., 1989; Kennedy and Limbird, 1993; Ng et al., 1994). The half-life of palmitate can vary from minutes to days. When protein synthesis is inhibited, palmitoylation may occur several hours later (Olson and Spizz, 1986). The enzymology of palmitoylation is not so well understood as that of myristoylation.

In contrast to prenylated and myristoylated proteins, which are found in the cytoplasm and the membrane, palmitoylated proteins are virtually always membrane-bound and palmitoylation is found in both integral and peripheral membrane proteins. Many proteins are modified by myristoyl or prenyl moieties before palmitoylation. These modifications increase the hydrophobic interactions between the protein and the lipid bilayer of the cell membranes (Casey, 1995).

Lipid Modification	Position of modification	Consensus sequence	Linkage formed
Prenylation Farnesylation Geranylgeranylation	 COOH-terminus COOH-terminus	 -CAAX or -CC or -CXC	 Thioether linkage Thioether linkage
Myristoylation	NH2-terminus	Glycine at position 2	Amide bond
Palmitoylation	Internal	Variable	Thioester linkage

Table 4. Three major lipid modifications of proteins.

A large number of neuronal (and also peripheral) proteins are known to be palmitoylated. The generality of this modification in nervous systems indicates its importance. For example, several ion channels, neurotransmitter receptors, and cell adhesion components are palmitoylated. The cycle of palmitoylation and depalmitoylation of signalling proteins is thought to be an important step in the regulation of signal transduction (Bizzozero et al., 1994). Proper palmitoylation plays a crucial role in the functioning of the nervous system, since palmitoylated and depalmitoylated proteins have different properties. The exact intracellular site where protein is palmitoylated (after it has been transported from the trans-Golgi network) has not been identified. Synaptic vesicles contain all the components for palmitoylation of SNARE protein. However, it has been demonstrated that, for example, the palmitoylation of SNAP-25 is dependent on a functional secretory pathway (Gonzalo and Linder, 1998).

One possibility, when considering how the lack of entirely different known NCL proteins cause disorders with the same clinical features, is that all NCL-encoded proteins are directly or indirectly linked to the same trafficking pathway (Zhong, 2000). If this is the case, it might be that some of these interactions are formed via lipid modifications, which are known to change the location, function, and conformation of proteins. For example, the CLN3 protein has myristoylation and prenylation sites (Kaczmarski et al., 1999).

3.3 Thioesterases

Thioesterases are enzymes that hydrolyse the thioester bond between the carboxyl end of the fatty acid and the sulfhydryl group of the cysteine residue of the protein. Identification of the molecular machinery responsible for acylation and deacylation of intracellular proteins has been difficult. Of the mammalian enzymes that remove palmitate from palmitoylated proteins, the first to be purified was PPT1 (Camp et al., 1994). Two other enzymes have been described: PPT2 is a lysosomal thioesterase with distinct substrate specificity than PPT1. This enzyme removes palmitate from palmitoyl CoA, but not from palmitoylated protein substrates. The chromosomal localization of the PPT2 gene is 6p23. At the amino acid level, the identity of human PPT1 with PPT2 is 20% (Soyombo and Hofmann, 1997). The third enzyme with deacylating activity is acyl protein thioesterase 1 (ACP1), a cytosolic protein which, *in vitro*, catalyzes the depalmitoylation of the heterotrimeric G protein α subunit and p21ras (Duncan and Gilman, 1998).

3.3.1 Palmitoyl protein thioesterase

Originally, PPT1 (E.C.3.1.2.22) was purified from bovine brain cytosol (Camp and Hofmann, 1993; Camp et al., 1994). PPT1 is a depalmitoylating enzyme that removes palmitate from the cysteine residues of acylated proteins such as H-ras. The enzyme

hydrolyses the thioester bond between the carboxyl end of the fatty acid and the sulfhydryl group of the cysteine residue.

The gene defect in INCL was mapped on chromosome 1p32 (Järvelä et al., 1991). The mutations responsible for INCL were identified in a gene coding for PPT1 (Vesa et al., 1995). The human PPT1 gene consists of 9 exons, covering 25 kb of genomic DNA. The size of the messenger RNA is 2.4 kb and it encodes a polypeptide of 306 amino acids having a signal sequence of 25 aa in the N-terminus. The PPT1 has three glycosylation sites and two sequence motifs that are characteristic of thioesterases (Schriner et al., 1996) (Fig. 7). Because of its heterogeneous glycosylation, PPT1 appears on the SDS-PAGE gel as a 39/37 kDa doublet. By mutagenizing the glycosylated asparagine to glutamine (Bellizzi et al., 2000), have shown that proper glycosylation is required for the activity and/or stability of the PPT1 enzyme. The triple glycosylation mutant has no detectable PPT1 activity.

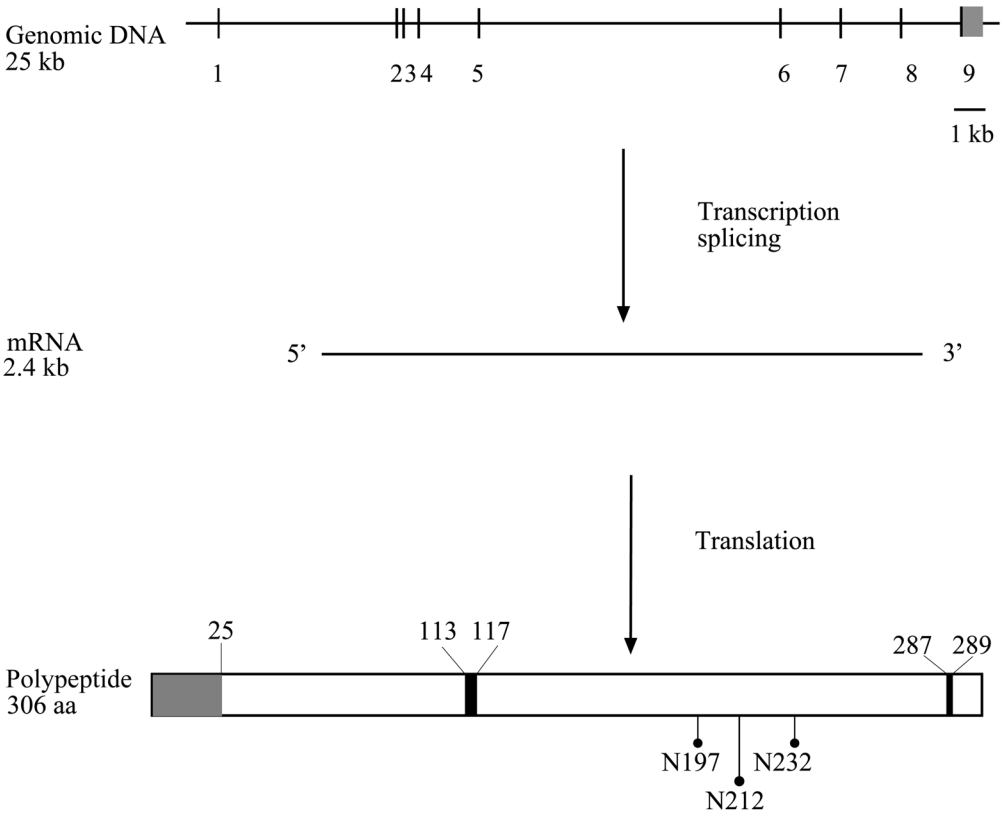

Figure 7. The structure of the PPT1 gene and its polypeptide.
The PPT1 gene is spanning a 25 kb region of the genomic DNA. The size of the messenger RNA is 2.4 kb. The signal sequence of the PPT1 polypeptide is consisting of 25 amino acids. Thioesterase sequences (113-117 and 287-289) are shown. Also three N-glycosylation sites are marked.

Earlier studies in nonneuronal cell lines have shown that PPT1 is a soluble lysosomal enzyme, synthesized by membrane-bound ribosomes and translocated into the lumen of the ER with the aid of the signal sequence. This signal sequence is cleaved in the ER. PPT1 is targeted to lysosomes via the M6P receptor-mediated pathway (Hellsten et al., 1996; Verkruyse and Hofmann, 1996). In an analysis of fibroblasts from patients with I-cell disease (the lysosomal enzymes of these patients lack the mannose-6-phosphate marker), the intracellular levels of PPT1 were dramatically reduced, but the levels of PPT1 in the culture medium were notably increased (Verkruyse et al., 1997). When immortalized lymphoblasts of INCL patients were labeled with [^{35}S] cysteine, an accumulation of [^{35}S] cysteine-labeled lipids was observed. *In vitro,* these lipids are substrates for PPT1 and their accumulation was reversed by addition of PPT1 to the cell culture medium (Lu et al., 1996). On the basis of these results, INCL has been classified as a classical lysosomal storage disorder.

PPT1 is a ubiquitously expressed enzyme, being most abundantly expressed in the spleen, brain, lungs, and testis (Camp and Hofmann, 1993; Suopanki et al., 1999). However, the highest activity levels of the enzyme have been found in the brain and the testis. The reason for this difference is not currently known. In the mouse and the rat brains, the expression of PPT1 is under developmental control. PPT1 expression increases during the maturation of central nervous system and the maximal level is reached in early adulthood. This expression is temporarily and spatially equal to synaptogenesis (Isosomppi et al., 1999; Suopanki et al., 1999). In cultures of mouse cortical neurons infected with the PPT1-adenovirus, PPT1 colocalizes with presynaptic synaptophysin and the synaptic vesicle marker SV2 (Heinonen et al., 2000). Furthermore, cell-fractionating studies of mouse brain tissues showed that PPT1 is located in the synaptosome and synaptic vesicle fractions but not in the lysosomal fraction (Lehtovirta et al., 2001). The recent finding that PPT1 is not enriched in the lysosomes of neurons may indicate a different function for PPT1 in the neurons. *In vitro*, PPT1 has been shown to depalmitate several neuron-specific peptides, for example, GAP-43 (growth-associated protein) and rhodopsin. Different substrates showed a different pH profile (Cho et al., 2000). In addition, the overexpression of the PPT1 reduces the membrane association of GAP-43 (Cho and Dawson, 2000).

The PPT1 enzyme has been crystallized and the structure is consisting of six paralled β-sheet altering with α–helixes (Fig. 8). The catalytic active site residues in PPT1 are Ser115, His289 and Asp233. Serine 115 is the catalytic nucleophile and is modified by palmitate (Bellizzi et al., 2000).

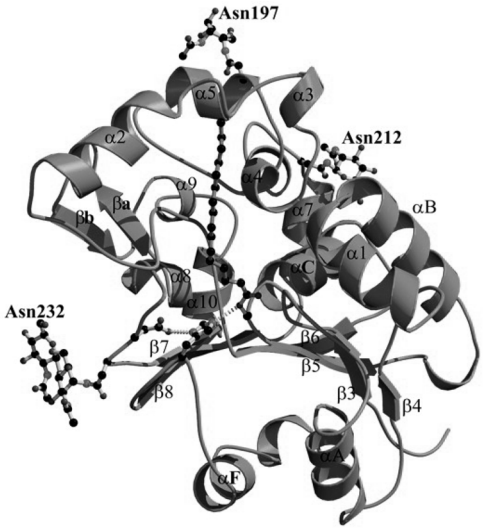

Figure 8. The crystal structure of the PPT1 protein.
This picture is reproduced with permission from "The crystal structure of palmitoyl protein thioesterase 1 and the molecular basis of infantile neuronal ceroid lipofuscinosis" by Bellizzi JJ 3rd, Widom J, Kemp C, Lu JY, Das AK, Hofmann SL, Clardy J. Published in Proc Natl Acad Sci U S A. 2000 Apr 25;97(9):4573-8. Copyright (2000) National Academy of Sciences, U.S.A.

3.3.1.1 PPT1 mutations and variable INCL phenotypes

In INCL the fine structure of predominant accumulating storage material is GROD (Haltia et al., 1973a). In Finland, 98% of the disease chromosomes are affected by a single missense A364T mutation in the PPT1 gene, resulting in an amino acid substitution Arg122Trp. The carrier frequency of this substitution, which causes complete inactivation of the PPT1 enzyme, is 1:70 (Vesa et al., 1995). The frequency of this mutation is remarkably much lower outside Finland, but it has been found in the United States in patients of Scandinavian origin (Das et al., 1998). The phenotype of all the Finnish patients is classical early-onset INCL.

The first clinical description of a juvenile patient with accumulating GRODs appeared in 1973 (Carpenter et al., 1973). Since then, several patients have been described resembling late infantile or juvenile phenotypes, with GROD instead of curvilinear or fingerprint bodies (Hofman and Taschner, 1995; Philippart et al., 1995; Lake et al., 1996; Crow et al., 1997). These subtypes are named vLINCL/GROD or JNCL/GROD

(Mitchison et al., 1998). About 50% of the PPT1-deficient patients in the United States have the classic, rapidly progressive INCL phenotype. Many of these patients are homozygous for a C451T transition, causing a premature stop codon at amino acid position 151 (Arg151Stop), as this allele accounts for 40% of all alleles in the U.S. population. About 20% of the patients that do not fit the classical phenotype develop the first symptoms between 2 and 4 years of age. The main mutation of this vLINCL phenotype is Gln177Glu (C529G) (Das et al., 1998; Waliany et al., 2000). The remaining 30% of PPT1-deficient patients have the juvenile-like phenotype, the age of onset being 5 years or later. A cluster of juvenile NCL/GROD patients has been described in Scotland (Mitchison et al., 1998). All the patients were carrying the Thr75Pro mutation. The American patients with the same mutation have Scottish ancestry. The Thr75Pro mutation accounts for most of the vJNCL/GROD cases. Patients with this mutations have 2-4% of residual enzyme activity. Recently, an ANCL patient with PPT1 deficiency was described. The age of onset was 38 years (the present age of the patient is 54). The mutation in one allele of this patient is Arg151STOP. The causative novel mutation in the other allele is Gly108Arg (van Diggelen et al., 2001) (Fig. 9).

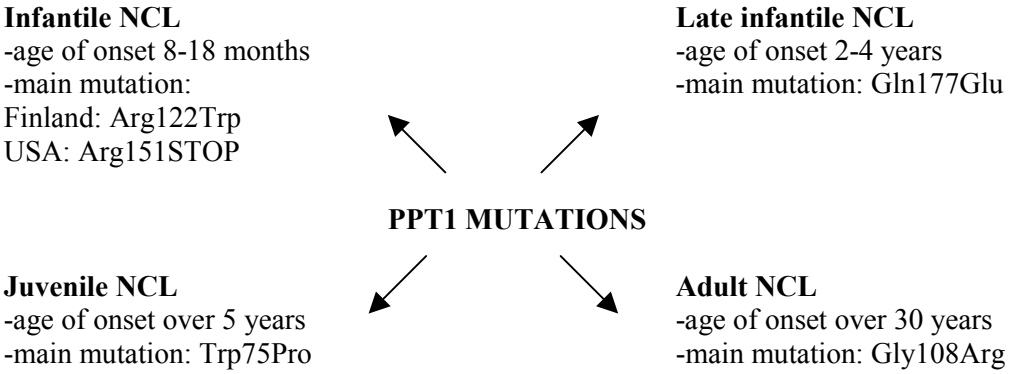

Figure 9. Mutations in the PPT1 gene cause different NCLs with varying age of onset.

The three dimensional structure of the PPT1 enzyme enables the analyses of the phenotype- genotype correlations (Bellizzi et al., 2000). The mutations affecting the classical severe phenotype are located near the active site serine (Ser155) disturbing the structure and function of the enzyme. The mutations causing the protracted phenotype are remote from the catalytic triad and lead to small local changes in the protein structure (Bellizzi et al., 2000).

AIMS OF THE PRESENT STUDY

This study was carried out in order to analyze the molecular genetics of novel PPT1 mutations causing INCL. Consequently, it was important to analyze the consequences of the PPT1 mutations at cellular level. To elucidate the disease-causing mechanism of INCL, the consequences of different PPT1 mutations on the neurons was also monitored. Characterization of the mouse PPT1 gene was also performed. This will enable targeted disruption of the mouse PPT1 gene when generating a knock-out mouse.

The specific aims were:

1. To identify novel PPT1 mutations causing INCL with the classical phenotype (I)

2. To monitor how PPT1 mutations causing the mild and the classical INCL phenotype affect the processing and localization of PPT1 in nonneuronal and neuronal cells (II)

3. To characterize the nucleotide sequence and the chromosomal localization of the mouse PPT1 gene. To clarify the intracellular maturation and localization of the mouse PPT1 polypeptide (III)

MATERIALS AND METHODS

Patients: The family material for the mutation analyses consisted of 11 patients. The origin of the patients' ancestors were: Austrian, Asian-Afro-Caribbean, English, French, German, Irish, Japanese, Turkish, and U.S. All the patients presented the classical severe INCL, the age of onset being 8-18 months and the electron microscopic ultrastructure of the storage material in a tissue biopsy showed GROD. Fifty healthy Caucasian control individuals were also included to exclude the possibility that a newly found mutation was a polymorphism. (I)

DNA and RNA extraction: Genomic DNA was extracted from peripheral blood samples as described earlier (Vandenplas et al., 1984) (I). Total RNA was extracted from mouse brain and liver tissues by the guanidium-isothiocyanate/ cesium chloride gradient method (Chirgwin et al., 1979) (II).

Exon amplification: In all PCR reactions, one primer was 5'-biotinylated. Primers were designated according to the intronic sequences of human PPT1 flanking each exon at a distance of 40 bp or more from the exon/intron boundary (Schriner et al., 1996) (I).

DNA sequencing: Amplified exons were immobilized on avidin-coated microparticles (Fluoricon IDEXX) and sequenced by the dideoxy chain termination method (Sanger et al., 1977) (I). Mouse PPT1 exonic sequences and exon/intron boundaries and the expression constructs used for the mouse PPT1 study were also sequenced by the dideoxy chain termination method (III). The sequencing of the SV-poly vector (Stacey and Schnieke, 1990) and recombinant Semliki Forest virus encoding wt or mutated PPT1 cDNAs was performed with an ABI prism 377 DNA sequencer (Perkin-Elmer Applied Biosystems) according to the BigDye Terminator cycle sequencing protocol (Perkin-Elmer) (II).

Site directed mutagenesis: The coding region of PPT1 (Schriner et al., 1996) was used as a template to create constructs containing naturally occurring INCL mutations. The selected mutations were A223C, T656A, 249-251delCTT and 132-133insTGT which were introduced into the wild-type PPT1 cDNA in the SV-poly vector. Mutagenesis was performed using a site-directed mutagenesis kit (Stratagene) (II).

The expression plasmids: The human wild-type PPT1 and PPT_{Fin} coding regions were originally cloned to the SV-poly expression vector (Vesa et al., 1995). The mutagenized cDNAs were digested from the SV-poly vector and subcloned to the pCMV5 vector (Andersson et al., 1989) (II). The mouse PPT1 coding region was cloned to the pCMV5 expression vector (III).

Cell culture: African green monkey kidney cell line COS-1 (ATCC CLR1650), human cervical carcinoma cell line HeLa (ATCC CCL2) and Baby Hamster Kidney (BHK) cell line (ATCC CCL-10) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin and

streptomycin). To obtain mouse primary neurons, the telencephalic structures of E16 embryonic mouse brains were dissected as described previously (Heinonen et al., 2000).

Cell transfection: For transfection, cells were seeded one day before and grown to 80% confluence. The cells were transfected, using three different techniques. In the DEAE-dextran and chloquine transfection method (Sussman and Millman, 1984), COS-1 cells were transfected with 5 μg of mouse PPT1 cDNA in pCMV5-vector (III) or with 5μg of the recombinant pCMV5 constructs (II). HeLa cell were transfected with 5μg mouse plasmic cDNA constructs, using the lipofectamine (GIBCO) transfection method (Felgner et al., 1987)(III). BHK cells were transfected by FuGene 6 transfection system (Roche Diagnostics) (II). Lipofectamin and FuGene 6 transfected cells were fixed 48 hours after transfection. Mouse cortical neurons were transfected immediately after preparation with the delPhe84 cDNA construct, using the FuGene 6 transfection method. Cells were fixed with 4% buffered paraformaldehyde after incubation for 48 hours (III).

Pulse chase analysis and immunoprecipitation: Three days after transfection with DEAE dextran method, the cells were incubated for one hour with 100 μCi/ml of [^{35}S] cysteine and [^{35}S] methionine, followed by chase periods of different time periods. The culture medium and the cells were harvested, and metabolically radiolabeled PPT1 polypeptides were immunoprecipitated, using the polyclonal anti-human PPT1 antibody (Hellsten et al., 1996). The immunoprecipitated PPT1 polypeptides were separated in 11% SDS-polyacrylamide gel (Laemmli, 1970) and visualized by fluorography (II, III).

Semliki Forest viruses (SFV) and SFV infection of mouse cortical neurons: The SFVs encoding wild-type PPT1 and PPT_{Fin} had been constructed earlier (Kyttälä et al., 1998; Lehtovirta et al., 2001). The three mutant (T656A, A223C, 132-133insTGT) cDNAs were digested from the corresponding pCMV5 expression construct and subcloned to the BamHI restriction site of the pSFV1 vector. The virus stocks were prepared as described earlier (Olkkonen et al., 1994). Mouse cortical neurons cultured *in vitro* for 5 days were infected with recombinant Semliki Forest virus. Cells were incubated with the virus for 1 hour and, after removal of the virus were incubated for 8 hours in the original medium. These cells were fixed with 4% PBS-buffered paraformaldehyde.

Immunofluorescence staining: For immunofluorescence analysis, the lipofectamin transfected HeLa cells (III) and FuGene 6 transfected BHK cells (II) growing on coverslips were fixed in methanol 48 hours after transfection. Mouse cortical neurons, transfected with delPhe84 cDNA or infected with recombinant Semliki Forest virus, were fixed with 4% buffered paraformaldehyde (II). Nonspecific staining was blocked with 0.5% BSA in PBS, followed by addition of primary antibodies. Polyclonal rabbit anti-human PPT1 antibody and antibodies against different organelle markers were used as primary antibodies (Table 5). To allow the double labeling, the secondary

antibodies were conjugated with either Fluorescein (FITC) or Rhodamine (TRITC). The immunofluorescence was viewed with either Zeiss Axiophot immunofluorescence microscopy (III) or Leica confocal microscopy (II).

Antibody	**Detecting**	**Reference/ Origin**
Anti-human PPT	PPT	Hellsten et al., 1996
Lamp1	Lysosome	Gift from Dr. Vesa Olkkonen
LGP120 tail	Lysosome	Gift from Dr. Peter van der Sluijs
CTR-433	Golgi	Gift from Dr. Michel Bornens
PDI	ER	Stress Gen, Victoria BC, Canada
1D4B	Lysosome	Developed by J. Thomas August, the Developmental Studies Hybridoma Bank
SV2	Synaptic vesicle	Developed by Kathleen M. Buckley, the Developmental Studies Hybridoma Bank

Table 5. Primary antibodies used in this study.

PPT1 activity assay: The enzyme activity of six different PPT1 polypeptides (A223C, T656A, 249-251delCTT, 132-133insTGT, $PPT1_{Fin}$, and PPT1 wild-type) were measured from transiently transfected COS-1 cells. The activity assay is based on release of fluorescent 4-methylumbelliferone from the 4-methylbelliferyl-6-thiopalmitoyl β-D-glucoside (MU-6S-Palm-βGlc), the substrate for the PPT1 enzyme activity (van Diggelen et al., 1999) (II).

Isolation of mouse PPT1 Gene: Human PPT1 cDNA was used as a probe to screen the 129 SVJ mouse liver genomic library in λ FIX II vector (Stratagene). Positive subclones were sequenced and sequences of exons and exon/intron boundaries were determined. The sizes of the introns were determined by PCR amplification, using primers located in the flanking exons. The sequence of exon 1 was characterized by sequencing the PCR product amplified from the cDNA kidney library (III).

Fluorescence *in situ* hybridization (FISH): The chromosomal location of the mouse PPT1 gene was found by hybridizing the mouse metaphase chromosomes with the mouse PPT1 probe and parallel hybridizing with the λ clone specific for the COL15A1 gene (Hägg et al., 1997). PPT1 genomic probes were labeled with biotin-11-dUTP (Sigma chemicals) and the mouse chromosome 4-specific COL15A1-specific λ clone was labeled with digoxigenin-11-dUTP (Boehringer Mannheim).

RESULTS AND DISCUSSION

1. Characterization of novel PPT1 mutations (I)

When this project began, 23 different PPT1 disease mutations were known (Vesa et al., 1995; Das et al., 1998; Mitchison et al., 1998; Munroe et al., 1998; Santorelli et al., 1998; Mole et al., 1999). In Finland, one founder mutation, A364T (Arg122Trp), is responsible for 98% of the Finnish INCL chromosomes (Vesa et al., 1995). The PPT1 mutations are distributed throughout the coding region of the PPT1 gene, with no potential hotspot regions. The two most commonly found mutations outside Finland are A223C (Thr75Pro) (Mitchison et al., 1998) and C451T (Arg151STOP) (Das et al., 1998). We had 11 samples from patients of unrelated families in which the mutations were not characterized. All the patients in these families had INCL of the classic severe phenotype. To identify the mutations responsible for the disease, we PCR amplified the genomic DNA of all nine exons of PPT1, including 20-40 bp of the intron-exon junctions, in order to sequence the mRNA splice sites also. Analysis of the sequences revealed a total of 10 PPT1 mutations, eight of which turned out to be novel, i.e. previously unknown PPT1 mutations (Fig. 10).

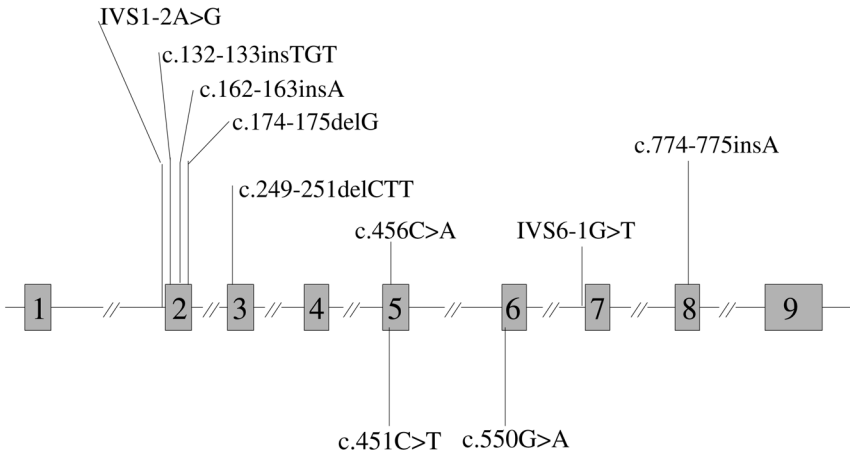

Figure 10. The PPT1 mutations identified in this study. Novel mutations are marked on the upper side of the PPT1 gene and previously reported mutations are marked on the lower side.

Two previously characterized mutations were C451T (Arg151STOP) (existing in 7 alleles out of the total of 20) and G550A (Glu184Lys) (2/20 alleles). In the U.S. population the most common INCL mutation is Arg151STOP, which accounts for 40% of the disease alleles and causes truncation of the polypeptide (Das et al., 1998). The studies of Das et al. have demonstrated that this early stop codon prevents the expression of PPT1 mRNA (Das et al., 2001). When the Arg151STOP mutation is homozygous the INCL is always a severe early-onset disorder. In the compound heterozygotes having Arg151STOP, the phenotype is either the classic or the juvenile-

like NCL with GROD, depending on the mutation in the other allele. In our material, this mutation was homozygous in an English patient, but in this case the disease was caused by maternal uniparental disomy (Louise Wilson et al. unpublished observation). In a Turkish patient whose parents where second cousins, a G550A (Glu184Lys) missense mutation was found to be homozygous. This mutations is located in exon 6 and had been reported earlier in a patient suffering from severe INCL and who had a Swedish ancestor (Das et al., 1998).

1.1 Mutations resulting in truncated polypeptides

Five of the novel mutations found in this study resulted in a premature STOP codon either directly or after a frameshift. One patient from Japan carried the nonsense mutation **C456A (Cys152STOP)**, which causes termination of the polypeptide after a normal 151 PPT1 amino acid. The mutation in the other allele of this patient was not found, indicating location of the mutation in the intronic sequences or in the promoter area.

One bp insertion in exon 8, **774-775insA**, was detected in a French patient. As a result of this mutation, the PPT1 polypeptide contained 258 amino acids of the correct PPT1 sequence followed by 34 novel aa caused by a frameshift before the STOP codon. This is the other patient in whom the nucleotide change in the other allele could not be detected. Since in both patients, the disorder is the severe early-onset type, it can be assumed that the undetected mutations, although not found in the coding region or in exon/intron boundaries, are located in the regulatory regions or in the introns and also give rise to a nonfunctional polypeptide. However, the promoter region of PPT1 has not been characterized, and, consequently the possible regulatory regions in the introns are so far unknown.

In a Turkish patient, a homozygous one bp insertion was found in exon 2. This **162-163insA** produced a PPT1 polypeptide that contained 54 normal N-terminal PPT1 amino acids followed by 46 nonsense amino acids. The size of this aberrant polypeptide was 100 amino acids.

A paternal mutation, **174-175delG**, was found in exon 2 of an English patient. This 1bp deletion also caused a frameshift with 58 wild-type amino acids and 10 nonsense amino acids. The other allele of this patient was the previously characterized Arg151STOP mutation.

The other Turkish patient had a homozygous splice acceptor **mutation IVS1-2A→G** in intron 1. A STOP codon was seen after 63 amino acids.

The mutations mentioned above all caused severely truncated polypeptides. In the cases of IVS1-2A→G, 162-163insA, and 174-175delG, all the amino acids belonging to the catalytic triad (Ser115-His289-Asp233) of the PPT1 enzyme were missing. In the case of the nonsense mutation A456C, the only remaining catalytic amino acid was

Ser115. It can be assumed that this would severely unstabilize the protein conformation, resulting in a nonfunctional polypeptide. A splice acceptor mutation was found in the paternal allele of a patient of English-Irish-German origin. This **IVS6-1G→T** splice acceptor site mutation in intron 6 most probably caused the skipping of exon 7, although exons 8 and 9 remained intact. The folding of this protein, like the folding of all the truncated proteins characterized here, would evidently be incorrect and, consequently, the mutant polypeptides would remain in the ER (Table 6).

Nucleotide change	Predicted consequence for the coding sequence/ Polypeptide level
IVS1-2A→G	Splice acceptor mutation in intron 1. 36 original PPT1 aa, STOP codon after 27 frameshift aa. Thioesterase sites and glycosylation sites deleted.
162-163insA	Insertion. 54 original PPT1 aa, STOP codon after 46 frameshift aa. Thioesterase sites and glycosylation sites deleted.
174-175delG	Deletion. 58 original PPT1 aa, STOP codon after the 10 frameshift aa. Thioesterase sites and glycosylation sites deleted.
456C→A	Cys152STOP. Polypeptide lacking the second thioesterase sequence, all glycosylation sites deleted.
IVS6-1G>T	Splice site acceptor mutation in intron 6. Skipping of exon 7.
774-775insA	Insertion. 258 original PPT1 aa, STOP codon after 34 frameshift aa. The second thioesterase site deleted.

Table 6. Predicted consequences of mutations causing truncation of the PPT1 polypeptide.

1.2 Mutations caused by deletion and insertion

In a patient whose parents were first cousins (Afro-Caribbean-Asian origin), a homozygous mutation was detected in exon 2. The **132-133insTGT** mutation led to insertion of an extra cysteine in position 45 of the PPT1 gene.

The mutation in one allele of the English-Irish-German patient was a three bp deletion in exon 3. This **249-251delCTT** resulted in deletion of phenylalanine 84.

Both of these mutations were found in an extremely conserved area of the PPT1 polypeptide. A reliable estimate of the consequences of these mutations for the PPT1 molecule is not possible. Therefore, these mutations were selected for further analysis,

in which, for example, intracellular trafficking was monitored in nonneuronal and neuronal cells.

2. Consequences of the mutations (I, II)

Some of the mutations previously found in the PPT1 gene produced a milder form of INCL (Das et al., 1998; Mitchison et al., 1998). In these patients the phenotype varied from late infantile to juvenile. The accumulating material showed GROD. About 30% of the PPT1 mutations characterized did not phenotypically resemble the classic early-onset INCL. In the INCL patients the most severe destruction is seen in the central nervous system. The organs most severely affected are the cortex of the brain and the retina. This study was carried out to monitor how PPT1 polypeptides with mutations causing either the classic or a protracted phenotype are processed in nonneuronal cells and localized in nonneuronal and neuronal cells. The selected mutations were: Thr75Pro (A223C) and Leu219Gln (T656A) resulting in a late-onset INCL phenotype and 132-133insTGT (insCys45) and 249-251delCTT (delPhe84) causing the classic INCL phenotype. Wild-type PPT1 was used as a normal control and PPT_{Fin} as a control for a severe phenotype.

2.1 PPT1 enzyme activity

Mutagenized coding regions and the wt PPT1 coding region were inserted into the pCMV5 vector, and COS-1 cells were transfected with these constructs. The PPT1 activities were measured from transiently expressed mutant polypeptides (Thr75Pro, Leu219Gln, delPhe84, ins Cys45, PPT_{Fin}) in COS-1 cells, using the non radioactive PPT1 activity assay (van Diggelen et al., 1999). It has previously been shown that, in patients homozygous for the PPT_{Fin} mutation, PPT1 enzyme activity in the brain tissue is undetectable (Vesa et al., 1995). The PPT1 activity in the Thr75Pro mutation was earlier measured in the immortalized peripheral blood lymphocytes and was found to be severely reduced, but residual activity of 2-4% could be detected (Das et al., 1998). It is possible that even this small amount of enzyme activity could cause the protracted INCL phenotype. In that study, the enzyme activity was measured using H-ras as a substrate (Camp and Hofmann, 1993). We used a fluorometric assay and demonstrated that the residual activity of the Thr75Pro-mutated PPT1 in transfected COS-1 cells was 5%. Surprisingly, no activity could be detected in PPT1 with the other late phenotype caused by the Leu219Gln mutation. PPT1 with the severe phenotype caused by the insCys45 mutation had 3% residual activity and the classic phenotype caused by delPhe84-PPT was totally inactive. In conclusion, the residual enzyme activity, when analyzed in transfected nonneuronal COS-1 cells, is not correlated with the severity of the disease, presumably due to the background endogenous PPT activity in the COS cells, which confounds the measurement of low levels of PPT activity.

The crystal structure of PPT1 has been characterized (Bellizzi et al., 2000) and has shown that PPT1 has an α/β-hydrolase fold. The Thr75Pro mutation is located in the α1-helix, remote from the catalytic triad. The geometry of the helix is perturbed by proline. Leu219Gln is also located in an α-helix and has been estimated to cause a

steric mismatch. The severe-phenotype-causing ins45Cys is located on a highly conserved region in exon 2. Native PPT1 has a disulfide bridge between the Cys45 and Cys46 residues; addition of an extra cysteine in this position most probably prevents the correct formation of disulfide bridge, resulting in an incorrectly folded polypeptide. The delPhe84 mutation is located in an α-helix in a region showing high conservation. Phe84 is in hydrophobic contact with Phe85. Deletion of the former phenylalanine most evidently destabilizes the structure of the PPT1 enzyme.

2.2 Intracellular processing

Earlier studies have shown that wild-type PPT1 appears as a doublet of 39kD and 37 kD in SDS-PAGE gels (Hellsten et al., 1996) and could be detected both in the cells and in the growth media. In order to study the intracellular processing of the mutations causing severe and milder phenotypes, the corresponding PPT1 polypeptides were transfected into COS-1 cells, labeled for one hour with [^{35}S] cysteine and [^{35}S] methionine, and chased for 0, 2, 4, 8, 12 hours and o/n. Cells were harvested and immunoprecipitated with anti-human PPT1 antibody and visualized by fluorography. All the mutant PPT1 polypeptides as well as the wild-type PPT1 and the PPT_{Fin} polypeptide, used as a severely mutated control, were matured equally to the 37 and 35 kDa forms. The glycosylation pattern found in each sample was similar to wild-type PPT1. The maturation studies in the peripheral COS-1 cells did not clarify the correlation between phenotype and genotype. The amount of PPT1 secreted was also the same for all the polypeptides. Whether PPT1 has an extracellular function or the secretion is due to an overexpression system remains unclear.

2.3 Distribution in BHK cells

In nonneuronal cell lines, it has been shown that wild-type PPT1 is transported to lysosomes, whereas PPT_{Fin} remains in the ER (Hellsten et al., 1996; Verkruyse and Hofmann, 1996). Confocal immunofluorescence microscopy of transfected BHK cells revealed that samples of PPT1 with mutations causing the classical phenotypes (insCys45, delPhe84, PPT_{Fin}) as well as those with protracted phenotype-causing mutations (Thr75Pro, Leu219Gln) all had similar staining patterns. In contrast to wt there was no colocalization with the lysosomal marker (LGP120), but colocalization with the PDI marker detecting ER could be observed. The only difference detected between the different mutations was that in the cells expressing the delPhe84 polypeptide, approximately every fifth transfected cell showed co-localization with the medial Golgi complex-specific (CTR433) antibody.

2.4 Intracellular trafficking of PPT1 in neuronal cells

In INCL, the most severely affected organ is the cerebral cortex, where there is rapidly progressive loss of neurons (Haltia et al., 1973b). Recently, it has been demonstrated

that, in mature neurons, PPT1 colocalizes with the presynaptic markers SV2 and synaptophysin. In contrast to the peripheral cells, colocalization with the lysosomal marker could not be detected. The cell-fractionating studies of the mouse brain gave identical results confirming this observation (Lehtovirta et al., 2001). To understand the phenotypic variations, the intracellular trafficking of mutant PPT1 polypeptides were also monitored in neuronal cells.

Delivery of genetic material into neuronal cells is difficult. The two systems most commonly used for expressing exogenous proteins in neurons are Semliki Forest viruses (SFV) and adenoviruses. SFV RNA functions as a messenger RNA in infected cells and consequently SFV cDNA is capable of protein synthesis. Mouse primary cortical neurons were infected with recombinant SFVs coding for wild-type and mutant PPT1 molecules. In spite of several repeated efforts, the construction of SFV encoding for the delPhe84 polypeptide was unsuccessful. Consequently, the localization of this polypeptide in mouse cortical neurons was monitored on transfected neurons. The transfection efficiency of delPhe84 was remarkably low. Moreover, only 15% of the neurons infected with SFV that were coding for mutated polypeptides and 10% of the neurons infected with SFV that were coding for wt PPT1 were infected. A low infection efficiency was also found when testing virus titers in BHK cells. The reason for the generation of unusually low titers of recombinant SFVs containing PPT1 is currently not known.

PPT1 with mutations causing the severe early-onset phenotype (insCys45 and delPhe84) colocalized with the ER-specific antibody PDI, while mutations causing the protracted phenotype (Thr75Pro and Leu219Gln) migrated along the neuritic shafts. These polypeptides colocalized with the synaptic SV2, protein but not with the ER-specific PDI protein (Table 7). The immunofluorescence puncta produced by mild mutations did not migrate quite as far from the soma along the neurites as the wild-type PPT1. However, this phenomenon could not be viewed experimentally with the existing equipment. Subcellular fractionation of infected neurons might have given a better view, but this was also too difficult to carry out because of the low infection efficiency and the large numbers of cells needed for subcellular fractionation.

Mutation	Phenotype	BHK cell localization	Neuronal localization
delPhe84	Classical INCL	80% ER 20% Golgi	ER
insCys45	Classical INCL	ER	ER
Thr75Pro	Protracted INCL	ER	Synaptic vesicle colocalization
Leu219Gln	Protracted INCL	ER	Synaptic vesicle colocalization

Table 7. Trafficking of different phenotype-causing mutations in neuronal cells.

Intracellular trafficking of PPT1 polypeptides in neuronal cells differs with the severity of the disease-causing mutation. For the first time, genotype-phenotype correlation could be shown in INCL. Probably, the severity of the disease is even influenced by low PPT1 enzyme activity in the synaptic areas. Measurement of the PPT1 enzyme activity in the neurons would give more accurate results, but because of the relatively low infection efficiency these measurements were too complicated to establish. The trafficking of PPT1 in the synaptic areas definitely does not follow the classical mechanisms known for endo- and exocytotic pathways in nonneuronal cells. The trafficking of PPT1 in neuronal cells and, overall, the whole protein trafficking in neurons is currently not understood in detail.

3. The mouse PPT1 gene (III)

3.1 Cloning the mouse PPT1 gene

The entire mouse PPT1 sequence was cloned in two steps. Genomic clones were isolated from a genomic library of the mouse strain 129 SVJ in the λ FIX II vector. Hybridization, using human PPT1 cDNA as a probe, resulted in isolation of two mouse PPT1-containing clones (spanning 20 kb of genomic DNA). Southern blot analysis revealed that each of these λ clones contained a sequence encoding exons 2-8, but that exon 1 was missing in both clones. These clones were digested with Sac I restriction endonuclease, subcloned into the pGEM4 vector, and sequenced with vector-specific primers as well as internal primers based on human PPT1 cDNA. To obtain the sequence of exon 1, a PCR reaction was performed with the mouse kidney cDNA library, using mouse PPT1 exon 4 (reverse) and a T7 primer from the vector library (forward). This library was also used to confirm the mouse PPT1 coding sequence. cDNA revealed an open reading frame of 918 bp coding for the 306 aa protein. The positions of the exon-intron boundaries were clarified by comparing the mouse genomic sequence with the mouse cDNA sequence obtained by RT-PCR (Fig. 11). When human and mouse PPT1were compared, the exon-intron boundaries were all identical. The exon sequences of cDNA and genomic DNA were identical in all but one nucleotide (378 is A in the mouse kidney cDNA and G in the genomic 129 SV DNA).

Figure 11. Genomic structure of the mouse and the human PPT1 genes.

Overall, on comparing mouse and human PPT1 genes, a high level of conservation was observed. Both genes have nine exons, mouse PPT1 spans 21 kb of genomic DNA whereas, because of the larger size of introns, the size of the human genomic counterpart is 25 kb (Schriner et al., 1996).

3.2 Chromosomal localization

Assignment of the PPT1 gene to the metaphase chromosomes was carried out by the FISH technique, with a pool of genomic subclones covering 10.5 kb of the mouse PPT1 gene as a probe. The COL15A1 gene has been mapped to chromosome 4B1-3 (Hägg et al., 1997) and was used as a reference clone to identify the mouse chromosome 4. The mouse PPT1 was mapped to chromosome 4 band D1-3, which is syntenic with human chromosome 1p32, where the human PPT1 is located (Järvelä et al., 1991).

3.3 cDNA and RNA analyses

A high level of conservation was observed when the mouse sequence was compared with the previously published human and rat sequences. At the nucleotide level 83.3% identity was detected between mouse and human and 94.1% between mouse and rat. Mouse PPT1 is 96.3% similar to human and 98.3% similar to rat PPT1 at the protein level. The degree of homology between these species is 100% in the acyl-CoA thioesterase consensus sequence. Untranslated 5' and 3' regions (46 bp and 1.5 kb) showed no conservation, suggesting no regulatory functions for these areas. Sequence analysis also revealed two potential polyadenylation sites. The first site was at nucleotide position 1288 and second 1 kb apart in the 3' direction.

RNA was prepared from eight mouse tissues and PPT1 expression was analyzed by Northern hybridization. This analysis showed that two transcripts, a major transcript of 2.65 kb and a smaller one of 1.85 kb, were present when hybridized with the probe containing mouse PPT1 cDNA. Mouse PPT1 is expressed as two transcripts, which arise by alternative polyadenylation. In all tissues except for the testis, the major transcript found was the 2.65 kb mRNA. The mRNA levels were quite equal in the different tissues, except in the testis and the kidney. In the kidney, the amount of the 2.65 kb transcript was clearly higher. The same amount of the transcript was also found in the testis, in addition to a 1.85 kb fragment, which was the major transcript in this tissue. Northern blot analysis of rat tissue RNA resulted in quite a different expression pattern (Camp et al., 1994). The highest levels of PPT1 mRNA were found in the spleen, the brain, the seminal vesicles and the testis, and the lowest levels were observed in the liver and the heart. The only correlation in the expression pattern was seen in skeletal muscle, in which the mRNA levels were lowest in both species. The choice of poly A site can affect to stability of the mRNA, the efficiency of translation, and the tissue-specific expression (Edwalds-Gilbert et al., 1997). PPT1 was expressed

in all the tissues examined both in human (Schriner et al., 1996) and in the mouse, suggesting that this enzyme has a housekeeping function.

3.4 Transient expression in COS-1 and HeLa cells

To monitor the intracellular processing and transport of the mouse PPT1 enzyme, the COS-1 cells were transfected with the CMV5 expression vector containing the cDNA encoding for the mouse PPT gene. In pulse chase analysis immediately after synthesis, four bands with apparent molecular masses of 32, 34, 36, and 38 kDa could be detected. Intracellular mouse PPT1 was processed to a mature 38/36 kDa form in a chase time of 2 hours. At this time point, the enzyme was also found extracellularly as a 38 and 36 kDa doublet. The size of mature human PPT1 was 37/35 kDa (39/37 according to Hellsten et al. (Hellsten et al., 1996). Consequently the mature mouse PPT1 enzyme migrates slightly slower on SDS-PAGE gels. Secretion into media is observed in both human and mouse PPT1 transfected cells, but the amount of extracellular PPT1 is greater in humans. It is also possible that extracellular PPT1 is caused by overexpression and does not correspond to the situation *in vivo*. Both mouse and human PPT1 have three potential N-glycosylation sites. Endo H treatment yielded a 32 kDa single polypeptide, suggesting that different polypeptide forms are caused by differential glycosylation. The slower migration is probably due to a difference in amino acid composition.

To characterize the intracellular localization of the mouse PPT1 polypeptide, HeLa cells were transfected with pCMV5 vector containing the coding sequence for the mouse PPT1 gene. In immunofluorescence analysis, the PPT1 showed colocalization with the lysosomal lamp1 marker, detecting the lysosomal and the early endosomal regions. Thus, as in to its human counterpart, mouse PPT1 is seen in the lysosomes of a peripheral cell line.

CONCLUSION AND FUTURE PROSPECTS

In order to clarify disease mechanisms, it is first important to analyze the gene product and the intracellular trafficking of the wild-type protein. Characterization of novel mutations makes it possible to offer direct mutation analysis based on prenatal DNA diagnosis in families in which such mutations are known. Polypeptides containing disease-causing mutations also need to be characterized at the cellular level. Only then is it possible to analyze the disease mechanisms in animal models. This study describes eight novel INCL mutations in the PPT1 gene. All the mutations identified were associated with the classic severe phenotype. Six of these mutations caused truncation of the PPT1 polypeptide and most probably these polypeptides are inactive and retained in the ER. The consequences of one amino acid insertion and of a deletion mutation (insCys45 and delPhe84) located in the conserved area of the PPT1 polypeptide could not be accurately estimated and, therefore, these mutations were selected for further analyses. Mutations causing the late infantile and juvenile phenotypes have also been described in the U.S. population (Das et al., 1998; Mitchison et al., 1998), and we were interested to monitor whether any phenotype-genotype correlations could be evaluated at the cellular level. The effects of two mutations causing the severe phenotype and two mutations causing the protracted phenotype were monitored in neural and nonneural cells. In the nonneural cell lines, all the mutant PPT1 polypeptides were mislocalized in the endoplasmic reticulum. The wild-type protein localized to the lysosomes. All of the mutant proteins were similarly defective in enzymatic activity, whereas the intracellular maturation was similar to that in the wild-type PPT1. Interestingly, in the primary neurons, the proteins with severe mutations resided in the ER. The PPT1-deficient molecules causing the mild phenotype were transported along the neurites and colocalized with the synaptic markers, similarly as the wild-type PPT1. Monitoring different types of mutations at the cellular level will also give further information on the normal function of PPT1. We found that the intracellular localization of the mutant PPT1 polypeptides in the neuronal cells is correlated with the disease phenotype. This is an important finding when characterizing the molecular mechanisms of INCL pathogenesis.

In this study, we also described the characterization of the mouse PPT1 gene. Furthermore, the lysosomal localization of the mouse PPT1 polypeptide in nonneuronal cells was shown. The next step in studying the disease mechanism of INCL was to use the characterized mouse PPT1 genomic DNA clones for the production of embryonic stem cells harboring a heterozygous targeted mutation in exon 2 of the PPT1 gene. Using this stem cell line, production of homozygous PPT1-deficient mice was unsuccessful, since no viable homozygous knock-out mice were born. Alternative approaches to produce the knock-out mouse model would be to mimick the human mutation, as was actually done in the case of aspartylglucosaminuria mice (Jalanko et al., 1998). As an alternative to the live mice, the ES cells could be used to knock out the other allele and render these ES cells homozygous. Such ES cells could then be differentiated into neurons and monitor differences in neuronal induction, development, and maturation.

Studies in experimental animals and in humans have shown that the expression of PPT1 starts already in the embryo and is developmentally regulated. The accumulation of storage material is seen in all the tissues, but the major clinical manifestations are found in the central nervous system. It is not known why PPT1 deficiency leads to selective death of cortical neurons. Interestingly, the expression pattern of PPT1 parallels synaptogenesis both temporally and spatially. Both in the brain and in neuronal cultures, PPT1 is transported along the neuritic shafts and colocalizes with the synaptic markers. However, the exact intracellular site of the depalmitoylation caused by PPT1 or its *in vivo* substrate(s) needs to be identified. The recent data on the neuronal trafficking of the PPT1 indicate that PPT1 is a component of a neuron-specific trafficking pathway. At the moment, it seems that the neuronal *in vivo* substrate for PPT1 should be looked for in the presynaptic terminals.

Currently, eight genetic loci underlying the NCL disorders have been identified (although most probably even more exist). It has been proposed that all NCL protein products have either direct or indirect interactions with each other. Consequently, all these proteins would participate in the same trafficking pathway. This hypothesis remains to be established.

In the future, the putative knock-out mouse model for INCL will enable studies of molecular alterations in the neurons and especially in the synapses. Furthermore, naturally occurring as well as knock-out animal models will have an important role in the development and testing future therapies for INCL. The ultimate challenge is to stop the neurodegeneration in this fatal disorder.

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