STUDIES ON PALMITOYL-PROTEIN THIOESTERASE 1

Implications for synaptic functions

Jaana Suopanki

University of Helsinki
2002

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Academic dissertation

To be publicly discussed with the permission of the Faculty of Science of the University of Helsinki, in the Auditorium 2041 Viikinkaari 5, Helsinki on Friday the 10th of 5, 2002 at 12 o'clock noon.

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ORIGINAL PUBLICATIONS

I Suopanki J, Tyynelä J, Baumann M, Haltia M. Palmitoyl-protein thioesterase, an enzyme implicated in neurodegeneration, is localized in neurons and is developmentally regulated in rat brain. Neurosci Lett. 1999, 265:53-6.

II Suopanki J, Tyynelä J, Baumann M, Haltia M. The expression of palmitoyl-protein thioesterase is developmentally regulated in neural tissues but not in nonneural tissues. Mol Genet Metab. 1999, 66:290-3.

III Suopanki J, Partanen S, Ezaki J, Baumann M, Kominami E, Tyynelä J. Developmental changes in the expression of neuronal ceroid lipofuscinoses- linked proteins. Mol Genet Metab. 2000, 71:190-4. Review.

IV Suopanki J, Lintunen M, Lahtinen H, Haltia M, Panula P, Baumann M, Tyynelä J. Status epilepticus induces changes in the expression and localization of endogenous palmitoyl protein thioesterase 1. Accepted to Neurobiology of Disease.

ABBREVIATIONS

aa	amino acid
AMPA	DL-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
BSA	bovine serum albumine
Ca^{2+}	calcium
CLN1	infantile neuronal ceroid-lipofuscinosis
CLN2	late-infantile neuronal ceroid-lipofuscinosis
CLN2p	CLN2 protein = tripeptidyl peptidase 1 pepstatin-insensitive proteinase
CLN3	juvenile neuronal ceroid-lipofuscinosis
CNS	central nervous system
CONCL	congenital ovine neuronal ceroid-lipofuscinosis
Da	dalton
DAB	diaminobenzidine tetrahydrochloride
DNA	deoxyribonucleic acid
E11	embryonic day 11
EM	electron microscopy
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate
GAP-43	growth-associated protein 43 kDa
GRODs	granular osmiophilic deposits
Hepes	N-(2-Hydroxyethyl)piperazine-N'(2-ethanesulfonic acid)
INCL	classic infantile neuronal ceroid-lipofuscinosis
KA	kainic acid
kDa	kilodalton
LINCL	classic late-infantile neuronal ceroid lipofuscinosis
LTD	long-term depression
LTP	long-term potentiation
Man 6-P	mannose 6-phosphate
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NCL	neuronal ceroid-lipofuscinosis
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
Palmitoyl-CoA	palmitoyl-Coenzyme A
P15	postnatal day 15
PBS	phosphate-buffered saline
PET	positron emission tomography
PPT1	palmitoyl-protein thioesterase 1
PSD	postsynaptic density
PSD-95	postsynaptic density protein, 95 kDa
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SAP90	synapse-associated protein, 90 kDa
saposins	sphingolipid activator proteins
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNAP-25	synaptosomal-associated protein 25 kDa
TBS	tris buffered saline
TPP-I	tripeptidyl peptidase I
TRITC	tetramethylrhodamine isothiocyanite

INTRODUCTION

A storage disease, a lysosomal disease, a lysosomal storage disease, a neurodegenerative disease, and a progressive encephalopatia are definitions applying for infantile neuronal ceroid-lipofuscinosis, INCL (CLN1). It is one of the most severe of the inherited diseases affecting children worldwide. Clinically, genetically, and pathologically INCL has been well characterized. However, due to difficulties in studying the developing human brain, investigations of the pathogenesis and mechanisms causing INCL have progressed slowly.

Normal development of an INCL-child during the first year of life is followed by dramatic and rapid deterioration of the central nervous system (CNS). Since the onset of INCL appears while synaptogenesis is ongoing, the mechanisms halting the normal infantile progress most possibly are involved in forming or maintaining neuronal connections. The defective protein causing this devastating disease is a lysosomal enzyme, palmitoyl-protein thioesterase 1 (PPT1). PPT1 is responsible for removing fatty acids from proteins *in vitro*. A single error in the PPT1 gene causes a progressive accumulation of proteins and lipids in neurons and formation of storage material, which blocks normal functions of the developing brain. So far, 37 mutations have been found to lead to the malfunction of the PPT1 enzyme. A natural, neuron-specific substrate of PPT1 is still missing and the physiological function of PPT1 remains a mystery.

The aim of the present thesis is to shed light on the developmental expression pattern of the PPT1 gene and protein. Our results were compared to two other lysosomal proteins tripeptidyl peptidase I, and cathepsin D, known to be involved in NCL-disease. Furthermore, the *in vivo* model of kainate-induced excitotoxicity in rat brains was used for seeking clues for the function of PPT1 in CNS neurons. The effects of kainate-induced status epilepticus and ongoing hyperexcitation of certain neurons to PPT1 expression and localization were examined.

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REVIEW OF THE LITERATURE

1. Neuronal ceroid-lipofuscinoses (NCLs)

The neuronal ceroid-lipofuscinoses (NCLs) are among the most common hereditary neurodegenerative disorders of childhood. They are autosomally recessively inherited. Worldwide incidence ranges from 0.2 to 7 per 100 000 live births (e.g. Rider and Rider 1988, Claussen et al. 1992, Cardona and Rosati 1995, Uvebrant and Hagberg 1997). NCLs show a characteristic, progressive accumulation of autofluorescent hydrophobic material, the so-called ceroid-lipofuscin, in the cytoplasm of neurons and to a lesser extent in many other types of cells. Lipofuscin accumulates in neurons and other cells during aging; ceroid granules develop during various pathological conditions (e.g. Ivy et al. 1984). In the late 1960s, scientists named the disease after this storage material resembling ceroid and lipofuscin (Zeman and Dyken 1969).

1.1 Classification of NCLs

During the last three decades, NCL-diseases have been divided into different types based on the age of onset, clinical course, electron microscopic findings and neurophysiology. The recent reports of NCLs with mixed clinical and neuropathological findings, atypical of classical NCL-types, however, have created a need for reclassification (e.g. Wisniewski et al. 2001a). At present, eight different genes are linked to NLCs, but not all of them have been identified. Table 1 summarizes the NCL-classification based on the gene defects. Despite the worldwide occurrence, some types are more prevalent in certain populations and countries than in others. Examples are CLN1, CLN5, and CLN8 in Finland; CLN2 and CLN3 in the USA; and CLN3 in Sweden and Norway (e.g. Rapola 1993, Uvebrant and Hagberg 1997, Mole et al. 1999 & 2001).

Table 1: Neuronal ceroid lipofuscinoses

Type	Age of onset	Gene location	Protein	References
CLN1, INCL	Infantile, And later ages of onset up to adulthood	1p32	PPT1 (palmitoyl-protein thioesterase 1)	Santavuori et al. 1973, Vesa et al. 1995, Das et al. 1998, Mitchinson et al 1998, van Diggelen et al. 2001
CLN2, classic LINCL	Late infantile, and later ages of onset up to juvenile	11p15	TPP-I (tripeptidyl-peptidase I)	Jansky 1908, Bielchowsky 1913, Sharp et al. 1997, Sleat et al. 1997 & 1999, Vines and Warburton 1999
CLN5, Finnish variant	Late infantile	13q22	407 aa membrane protein, function unknown	Santavuori et al. 1982, Savukoski et al. 1994, 1998
CLN6, Czech variant	Late infantile	15q21 -q23	Unknown	Lake and Cavanagh 1978, Elleder et al. 1997, Sharp et al. 1997 & 2001
CLN7, Turkish variant	Late infantile	8p23	Unknown	Wheeler et al. 1999, Mitchell et al. 2001
CLN8, NE*	Late infantile	8p23	286 aa membrane protein, function unknown	Tahvanainen et al. 1994, Hirvasniemi et al. 1995, Ranta et al. 1999, Herva et al. 2000
CLN3, JNCL	Juvenile, classic	16p12	438 aa membrane protein, function unknown	Stengel 1826, Batten 1903, Spielmayer 1905, Vogt 1909, Sjögren 1931, Santavuori 1988, Eiberg et al. 1989, The international Batten disease consortium 1995
CLN4	Adult, Kufs disease/ Parry disease	-	Unknown	Kufs 1925, Berkovic et al. 1988

* Northern Epilepsy

1.2 Clinical features and neuropathological findings

All childhood NCLs share similar clinical features such as loss of psychomotor skills and vision, epileptic seizures, and mental decline. In addition to timing, severity and pattern of occurrence of the aforementioned features differentiate NCL-types clinically (Santavuori 1988, Rapola 1993, Santavuori et al. 1993).

The major neuropathological feature in NCLs is the selective loss of CNS neurons. In CLN1, the brain atrophy is the most extreme due to complete loss of neurons. Milder neuronal loss in CLN2 and CLN3 leads to less severe brain atrophy (*Figure 1*). Astrocytic proliferation and hypertrophy always accompany the neuronal loss, resulting in ruined brain architecture in CLN1 and CLN2. Cerebellum is also affected in CLN1 and CLN2 (Haltia et al. 1973b, Rapola 1993, Goebel 1995). In all childhood forms of NCL, the retina degenerates with an almost total loss of photoreceptor and ganglion cells (Tarkkanen et al. 1972, Goebel et al. 1974).
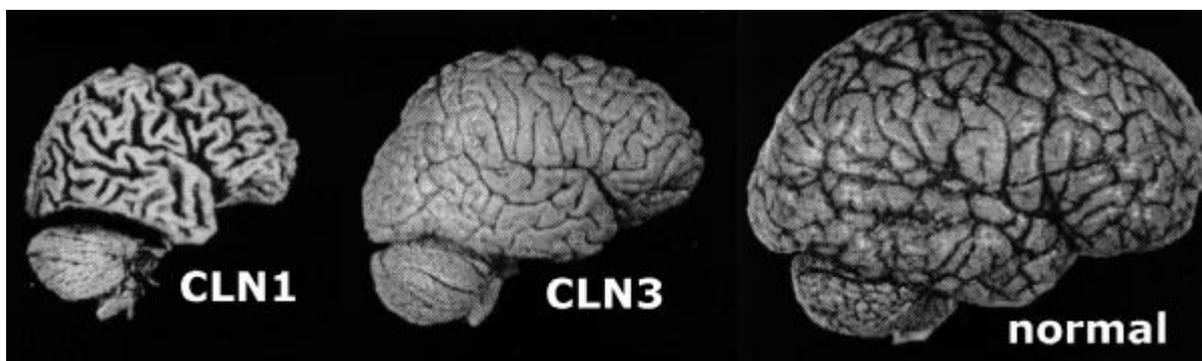
Figure 1. CLN1, CLN3 and normal brains in the same scale.

The accumulation of intralysosomal autofluorescent material, so-called storage cytosomes, is common in all NCL-types. The most prominent accumulation is detected in neurons, but other cells also show varying degree of storage (e.g. Haltia et al. 1973b, Rapola 1993). The main protein components in the storage material are either sphingolipid activator proteins A and D (saposins A and D; Tyynelä et al. 1993) or subunit c of mitochondrial ATP synthase (subunit c; Palmer et al. 1989, Hall et al. 1991). Saposins accumulate specifically in CLN1, while subunit c is found in the other NCL types. The ultrastructural pattern of the storage material is granular (CLN1), curvilinear (CLN2) or fingerprint-like (CLN3) (e.g. Haltia et al. 1973a, Carpenter 1977). In addition, mixed fingerprint/curvilinear/rectilinear patterns can be found in CLN5-CLN7 (Åberg 2001).

2. Infantile neuronal ceroid-lipofuscinosis (INCL, CLN1)

In 1973, Santavuori et al. published the first clinical and pathological description of infantile neuronal ceroid-lipofuscinosis (INCL, CLN1), the most severe of the NCL disorders. At that time, early diagnoses were based on brain biopsies (Haltia et al. 1987). Later, placental biopsies or rectal biopsies of children 2 months old provided diagnosis at the earliest disease stages (Rapola et al. 1984). Electron microscopic (EM) analysis of these tissue samples revealed ultrastructure of the typical granular osmiophilic deposits (GRODs) in storage cytosomes (Haltia et al 1973a, Rapola et al. 1984). At present, the main diagnosis is usually based on DNA analysis or on a fluorogenic PPT1 activity assay (Järvelä et al. 1991, Vesa et al. 1995, van Diggelen et al. 1999). Prenatal diagnosis is based on EM-analysis of chorionic villi (Rapola et al. 1990). Additionally, at an early stage of the disease, clinical diagnosis is done based on magnetic resonance imaging (MRI) findings alone, and biopsy is used only if unclear findings are observed (Santavuori et al. 2000). Up to date, 163 INCL patients have been diagnosed in Finland (Åberg 2001).

2.1 Clinical picture

The life span of INCL children can be divided into five clinical stages, stage 0: prenatal → 5 months of age, stage 1: 5 → 13 months of age, stage 2: 7 → 20 months of age, stage 3: 14 → 36 months of age, stage 4: from 2.1 years onwards (1- 4 originally described by Santavuori et al. 1993, 0- stage added by Vanhanen 1996). At **stage 0**, prenatal and early postnatal neurological development is normal up to approximately 5 months of age. Although the head may already be small at birth, the first sign of INCL is a decreasing head growth rate starting at the age of 5 months. MRI shows no changes or abnormalities in the brains of INCL children less than 6 months of age. During **stage 1** most of the INCL children learn to stand up and say some words. Only about 30 % of them learn to walk alone (Santavuori 1988). MRI images reveal affected white matter. During **stage 2**, overall development slows down. Cerebrocortical and cerebellar atrophy is evident by the age of 13 months, detected by MRI. A rapid decline continues at **stage 3**. Epileptic seizures appear at the mean age of 2.9 years. In **stage 4**, INCL children become blind, lose all cognitive and motor skills and become bedridden. The years 1-3 are often restless with disturbed sleep cycles, but terminal stage is usually peaceful. Death occurs between 9-11 years of age (Santavuori et al. 1973, Santavuori 1988, Vanhanen 1996, Santavuori et al. 2000).

At the moment, no cure or special preventive treatment is available for any NCL-type. The latest studies using lysomotrophic drugs for lysosomal ceroid depletion have raised

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new hopes for an effective treatment (Zhang et al. 2001, described further in the *Discussion*).

2.2 Neuropathological findings

Accumulation of GRODs in INCL-neurons begins early during the disease (Haltia et al. 1973a, Rapola 1993). GRODs have already been detected at 8 weeks of pregnancy (Rapola et al. 1990). Postnatally, moderate neuropathological changes appear in cortical neurons of patients up to 2.5 years, including neuronal destruction/loss and astrocytosis. During the period 2.5 – 4 years of age, loss of cortical neurons and astrocytosis progresses together with demyelination in the white matter. After 4 years of age, axons and myelin sheats have completely disappeared from the cerebral cortex (Haltia et al. 1973b).

The INCL brains are extremely atrophic, weighing about one fourth of the normal brain (page 9, *Figure 1*). At the terminal stage there are no neurons left in the cerebral cortex. Also, the cerebellar cortex and the retina are completely destroyed (Haltia et al. 1973b, Tarkkanen et al. 1977). Neurons remaining elsewhere in the CNS are full of storage material, which is also seen in many visceral and peripheral tissues (e.g. heart, intestines, kidneys, liver, skeletal muscles, skin) without any signs of cellular destruction (Haltia et al 1973b). The storage material consists of saposins A and D (Tyynelä et al. 1993) and lipid-thioesters from acylated proteins (Lu et al. 1996).

3. Palmitoyl protein-thioesterase 1 (PPT1)

In 1995, the defect responsible for INCL was identified in a gene encoding palmitoyl protein-thioesterase 1 (PPT1). One major mutation (Arg122Trp) was found in the Finnish population (Vesa et al. 1995). Since 1995, additional 36 PPT1 mutations have been identified, spanning every 9 exons of the gene (e.g. Mitchinson et al. 1998, Salonen et al. 2000, Santarelli et al. 1998, Waliany et al. 2000, Das et al. 2001). Patients with defective PPT1 can be found from Europe, the United States and Saudi-Arabia (Salonen et al. 2000, Hofmann et al. 2001). Thus, INCL is no longer solely a Finnish disease. Furthermore, the PPT1 mutations are associated not only with infantile but also with late-infantile, juvenile and adult phenotypes (e.g. Mitchinson et al. 1998, Salonen et al. 2000, van Diggelen et al. 2001, Hofmann et al. 2001). Therefore, PPT1 deficiency is clinically a very heterogeneous disease, which affects patients from different ethnic backgrounds (e.g. Das et al 2001, Hofmann et al. 2001). Common in all PPT1 deficiencies are the neurological symptoms, the GROD-type morphology in storage

cytosomes, and the accumulating saposins. But the phenotype or the severity of the disease development cannot be predicted from these features.

3.1 Structure

The cDNA of human, bovine, rat, or mouse *PPT1* is composed of eight coding exons and a large, ninth exon containing a 3′ untranslated region (Camp et al. 1994, Schriner et al. 1996, Salonen et al. 1998). The eight exons make up a coding region of 918 bp, which encodes 306 amino acids including a signal sequence of 25 (human)/ 27 (bovine, rat, mouse) amino acids. At amino acid level, human PPT1 (without the signal sequence) is 94% homologous to bovine PPT1 and 88% identical to rat PPT1 (Schriner et al. 1996). Bovine PPT1 is 82 % identical to rat PPT1 (Camp et al. 1994, Verkruyse and Hofmann 1996). Three potential asparagine-linked glycosylation sites, conserved among all these species, reside near the carboxyl terminus at positions 199, 214, 234. Nonglycosylated PPT1 has a size of 31 kDa. High mannose-type and complex asparagine-linked oligosaccharide modifications increase the size to 35-37 kDa (human) / 37-39 kDa (rat, bovine) (Camp et al. 1994, Verkruyse and Hofmann 1996, Schriner et al. 1996).

X-ray crystal structure analysis of bovine PPT1 shows a globular monomeric enzyme with a predicted α/β hydrolase fold and a catalytic triad of serine 115, aspartic acid 233, and histidine 289 (*Figure 2*). Correlations between the location of mutations and the predicted structural changes have been suggested to explain the alterations in PPT1 deficiency. Mutations affecting either catalysis or substrate binding or distorting proper folding of the enzyme core would lead to a severe phenotype with no enzyme activity. Less severe mutations causing local changes distant from the catalytic triad and palmitate-binding site would lead to a less severe disease, due to some residual activity (Bellizzi et al. 2000, Das et al. 2001).
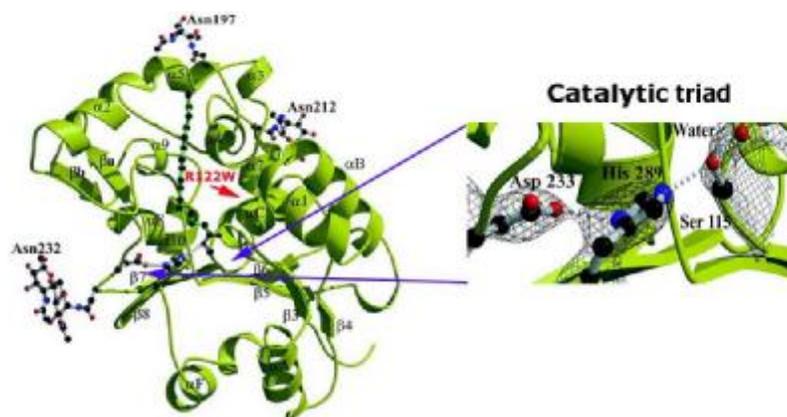
Figure 2. The crystal structure of PPT1 with palmitate. The glycosylated asparagines, the catalytic triad (shown also on the right) and the palmitate (in the middle) are indicated. The major INCL-mutation (Arg122Trp) is pointed in red. Bellizzi et al. 2000.

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3.2 Expression and function

PPT1 gene and protein expression was studied in selected rat and mouse tissues. PPT1 mRNA is highly expressed in lungs, spleen, pancreas, brain, seminal vesicles, and testis (Camp and Hofmann 1993, Camp et al. 1994, Schriner et al. 1996, Salonen et al. 1998). Expression in the brain was widely distributed with more prominent mRNA levels in neurons of the hippocampus and the cerebral cortex (Isosomppi et al. 1999). Liver, kidneys, heart, and skeletal muscles showed the lowest levels of PPT1 mRNA (Camp and Hofmann 1993, Camp et al. 1994). Protein expression levels in different tissues were never investigated. Instead, the ubiquitous expression of PPT1 in the brain was of major interest (Isosomppi et al. 1999, discussed further in this study).

Originally, PPT1 was purified from the bovine brain. It was shown to have a neutral pH optimum with broad substrate specificity. PPT1 removed fatty acids (acyl chains of 14-18 carbons) from cysteine residues of post-translationally lipid-modified proteins, such as S-acylated Ha-Ras (H-Ras, p21[Ras]), as well as from palmitoyl-CoA and palmitoylated neurospecific peptides, *in vitro* (Camp and Hofmann 1993, Cho et al. 2000b). Seventy to ninety per cent of PPT1 activity was found in cytosolic fractions, the rest resided in membrane fractions (Camp et al. 1993). PPT1 deacylating activity was significantly higher in the spleen, testes, and seminal vesicles than in the brain, which had the highest levels of mannose 6-phosphorylated PPT1 (Camp and Hofmann 1993, Sleat et al. 1996).

In fibroblasts or lymphoblasts, endogenous PPT1 was found in lysosomes and in the extracellular space. Transient expression of PPT1 showed that the recombinant protein was phosphorylated on mannose residues and transported to lysosomes via the Man 6-P receptor mediated pathway (Camp et al. 1994, Verkruyse and Hofmann 1996, Hellsten et al. 1996). The recombinant PPT1 was able to reverse the accumulation of lipid thioesters in INCL lymphoblasts *ex vivo* (Lu et al. 1996). In I-cell disease, where lysosomal enzymes are synthesized without the Man 6-P signal, the majority of lysosomal enzymes never reach their destination (Reitman et al. 1981, Kornfield and Sly, 1995). Also, the amount of intracellular PPT1 was reduced in I-cell disease fibroblasts. Moreover, the amount of extracellular PPT1 was highly increased in the growth medium (Verkruyse et al. 1997), indicating that PPT1 is a typical lysosomal enzyme, at least in nonneuronal cells.

Recent neuronal studies concerning PPT1 function showed that overexpressing PPT1 in neuroblastoma cells diminished palmitate-assisted binding of GAP-43 and p21[Ras] to membranes (Cho and Dawson 2000, Cho et al. 2000a). When this stable PPT1

overexpression was inhibited by antisense PPT1, the cells showed lowered resistance to apoptosis (Cho et al. 2000a).

3.3 PPT1 mutations *vs* disease phenotype

It has been suggested that nonsense and frameshift mutations in *PPT1* cause the severe classic infantile NCL, while missense mutations could be responsible for late-infantile or juvenile phenotypes (Das et al. 1998). Mutations leading to INCL were shown to correlate with a complete loss of PPT1 activity or absence of mRNA (Vesa et al. 1995, Das et al. 2001). Consistently, missense mutations responsible for late-onset phenotypes correlated with diminished PPT1 activity. Possibly, improper folding of the enzyme leads to the difficulties in substrate binding or alters enzyme stability (Das et al. 2001). Interestingly, a recent report described two French sisters (age 54 and 56 years) with adult NCL having causative mutations in *PPT1*. Their only symptoms were of psychiatric origin with onset in their fourth decade. Later, visual, verbal, and cognitive skills started to decline. EM examination of cutaneous biopsies showed GRODs in the sweat glands. DNA analysis revealed that the sisters supposedly had compound heterozygosity; both had a deleterious mutation in *PPT1* exon 5 and a missense mutation in exon 3. Furthermore, PPT1 depalmitoylating activity in both cases was in the range of *in vitro* activity measured from INCL fibroblasts or leukocytes (van Diggelen et al. 2001).

It has been suggested that the mutant PPT1 enzymes are retained in the ER and this would cause INCL (Hellsten et al. 1996, Das et al. 2001). Das and coworkers showed that the mutant enzymes had Man 6-P tags, but they did not bind to Man 6-P receptors *in vitro*. Thus, they suggested that oligosaccharide modifications were not properly trimmed (Das et al. 2001). A recent study compared nonneuronal overexpression to neuronal overexpression of mutated PPT1 enzymes. Intracellular PPT1 localization and the disease phenotype were found to correlate in neuronal cultures, but not in nonneuronal cultures (Salonen et al. 2001).

4. Other lysosomal NCL-proteins

4.1 Tripeptidyl peptidase I (TPP-I)

CLN2 protein was first isolated from the human brain and identified as a Man 6-P glycoprotein. The CLN2 gene had sequence similarities to a bacterial lysosomal protein, pepstatin-insensitive endoproteinase (named pepinase by Sleat et al. 1997, Oda et al 1994). Yet, later findings of Vines and Warburton (1998 & 1999) and Rawlings and Barrett (1999) demonstrated that the CLN2 protein is identical to lysosomal tripeptidyl

peptidase –I (TPP-I). Both aforementioned enzyme activities resided in the protein, but TPP-I activity was stronger and more evident than pepinase activity (Sohar et al. 2000).

Normally, the TPP-I gene and protein are expressed in various organs and tissues and, most importantly, in all types of brain cells (Sleat et al 1997, Kurachi et al. 2001, Kida et al. 2001). The adult expression pattern of TPP-I in the brain is reached at around 2 years of age (Kurachi et al. 2001, Kida et al. 2001), coinciding with onset of classic late infantile NCL (e.g. Rapola 1993).

In vitro substrate specificity of TPP-I is broad. As an exopeptidase TPP-I cleaves tripeptides from 4-42 residues long peptides with free N-termini (Junaid et al. 2000) and, most likely polypeptides of 4.5 kDa - 6 kDa can be degraded by TPP-I (Bernardini and Warburton 2001). Based on studies by Ezaki and coworkers, the protein accumulating in CLN2, subunit c, could be an *in vivo* substrate for TPP-I. Co-incubation of extracts from normal and CLN2 fibroblasts resulted in degradation of subunit c (Ezaki et al. 1997 & 1999). TPP-I mutations responsible for CLN2 phenotype are associated either with a diminished enzyme activity or complete loss of translated product (Sleat et al. 1997 & 1999, Sohar et al. 1999, Vines and Warburton 1999, Wisniewski et al. 2001b). To date, 40 mutations have been characterized in the TPP-I gene (Mole et al. 2001).

Recent overexpression studies showed that the majority of recombinant TPP-I was secreted as a soluble and inactive proenzyme of 65 kDa. In pH 3.5, it was converted to a 46 kDa form, which is an enzymatically active, mature form of TPP-I. Internalization into neurons or fibroblasts kept the mature form active for more than 10 days, and it was able to reverse subunit c storage (Lin and Lobel 2001a, b). As indicated by Lin and Lobel (2001b), the properties of TPP-I make the recombinant protein valuable for enzyme-replacement therapy.

4.2 Cathepsin D

A nucleotide change in cathepsin D gene causes congenital ovine neuronal ceroid-lipofuscinosis (CONCL), an inherited neurodegenerative disease of sheep. This disease has similar pathological findings to human NCLs (Järplid and Haltia 1993, Tyynelä et al. 2000). Due to the mutation, the active site aspartate of cathepsin D, an aspartic proteinase (reviewed by Conner 1998), is changed into asparagine, which leads to a stable but inactive enzyme. Activities of certain lysosomal enzymes, such as cathepsin C and TPP-I, are increased in the CONCL brain (Tyynelä et al. 2000).

Cathepsin D knockout mice were generated to enlighten in vivo functions and a physiological significance of the enzyme (Saftig et al. 1995). The mice developed normally until P20. Neurological symptoms including seizures and blindness were prominent at the terminal stage. Due to progressing atrophy of the intestinal mucosa, the mice died in an anorexic state, in approximately the fourth week of life (Saftig et al. 1995, Koike et al. 2000). A closer morphological investigation revealed that GRODs and fingerprint-like structures accumulated progressively in neuronal cytosomes of the cathepsin D deficient brain already after birth. Furthermore, subunit c of mitochondrial ATP synthase was the major component of the storage material. As expected from other lysosomal studies, the amounts of certain lysosomal enzymes and their activities (e.g. cathepsin B and TPP-I) were elevated in cathepsin D deficient brains (Koike et al. 2000). Therefore, cathepsin D knockout mice provide a new animal model for NCL-studies.

5. General aspects of brain development

5.1 The human brain

The most important developments in human brain structure occur during the first two years of life. Although the same sequential events are observed in animals, it is the slower time scale of these events and a larger volume of certain developing cerebral areas, particularly the frontal cortex, that differentiates the human brain development from other species. Even though humans are very dependent on parental care for a significant time after birth, the stage of neuronal development at birth is much more progressed relative to other species (e.g. reviewed in Clancy et al. 2000, 2001).

At the time of birth, most neurons have already migrated to reach their destinations within, for example, the hippocampus, the cerebral cortex, or the cerebellar cortex areas. Synaptogenesis, the forming of neuronal connections, progresses also rapidly in all cortical areas around the time of birth. Regional connections between different brain areas, however, are still very immature, mainly because different subcortical areas and cortical regions continue growing and developing at variable times after birth (e.g. Yamada et al. 1997 & 2000). Brain structures possibly reach the adult appearance by 2 years of age (e.g. Matsuzawa et al. 2001, Paus et al. 2001).

Positron emission tomography (PET) -studies have shown that sequential maturation of brain areas starts before the first month of age. This is demonstrated by the rising metabolic activities (=glucose uptake) in e.g. the sensorymotor cortex and brainstem. After 3 months of age, the cerebellum and different cerebral cortical areas, except the frontal cortex, show rising activities. After approximately 6-8 months of age, the frontal

cortex starts to mature (e.g. Chugani et al. 1987). As seen by MRI images, myelination of neuronal fibers begins at birth, has rapid changes during the first 2 years and continues throughout adolescence into adulthood (e.g. Paus et al. 2001). Overall metabolic activity has adult-like levels by the age of 9 (e.g. Chugani et al. 1987, Johnson 2001).

Brain weight reaches adult values between 10-12 years of age. The fastest growth occurs during the first 3 years of life and, by the age of 5 years, infants' brains weigh about 90% of adults (e.g. Dekaban 1978).

5.2 The rat brain

The gestation time for a rat is 21 days. Neurons of the cortical areas are mainly generated at embryonic day 16-21 (E16-E21) (e.g. Berry et al. 1964, Berry and Rogers 1965). Formation of neurons in the hippocampal region is also completed before birth (Bayer 1980), but there are variations among the areas of the hippocampus. For example, neurons of dentate gyrus continue to be formed until postnatal day 20 (P20). After E21 only the glial cell generation continues. In rats as in all mammals, the cerebral cortex is assembled slowly. At birth, the neurons in the deep cortical layers have arrived at their final positions, while those of superficial layers are still migrating until 4-7 days after birth (e.g. Berry at el. 1964, Hicks and D'Amato 1968). The cerebellum is relatively immature at birth. Thus, its histogenesis and morphogenesis occur mainly during postnatal development. Depending on the differentiating cell type, migration in the cerebellum continues approximately until P30 (e.g. Jacobson 1991).

5.3 Synaptogenesis

In the human brain, formation of dendritic trees and their synapses occurs during postnatal development. Around the time of birth, synaptogenesis progresses rapidly in all cortical areas, but synaptic maturation has a slower path. Furthermore, peak density of synapses and synaptic rearrangements vary in different areas at different ages. The major synaptic connections in the visual cortex are formed around 3-4 months of age. The maximum synaptic density, which is estimated to be 150% of the adult visual synapse levels, is reached by the first year of age. Although synaptogenesis in the prefrontal cortex starts at the same time as in the visual cortex, overall synaptic formation occurs much more slowly and peaks well after the first year (e.g. Rakic et al. 1986, Huttenlocher et al. 1979 & 1990). Though all major fibers can be detected already by the age of 3, the rise and fall of synapses (= synaptic pruning) in all cortical areas is estimated to reach adult levels during late childhood, (e.g. Matsuzawa et al. 2001, Paus et al. 2001).

In the rat brain, connections between nerve fibers develop in the late fetal and early postnatal periods (e.g. Ivy and Killacky 1982). There is a 10-fold increase in neuronal connectivity between P12-P30 in the rat cerebral cortex. Synapse formation in the cerebral cortex starts at birth and reaches a peak around P26. In the hippocampus, 90 % of adult synapses are formed at P30 (e.g. Eayrs and Goodhead 1959, Crain et al. 1973).

The specific molecular events leading to connections between presynaptic nerve terminal and postsynaptic neuron remain unsolved. Recent advances due to modern imaging techniques, which allow time-lapse observations of molecular movements even in intact animals, have helped to characterize and to define some of the synaptogenesis events. The formation of synapses is presumed to begin with elevated activities of one or both synaptic partners leading to a new physical contact. Naturally, this means that a postsynaptic cell could also initiate synaptogenesis. Sequential steps towards a synaptic connection are predicted to include the initial assembly of a highly specialized junctional cytoskeletal matrix that first stabilizes an adhesion site. Some studies have suggested that the adhesion proteins may make the first connections prior to any further events in synaptic formation. Whichever way this initiation occurs, it eventually promotes the recruitment and clustering of synaptic vesicles on the presynaptic side within the assembled cytomatrix proteins. This action allows formation of an active zone for synaptic vesicles, and a periactive zone for maintaining exocytosis and endocytosis activities. Scaffolding proteins, neurotransmitter receptors and ion channels cluster at the postsynaptic membrane (e.g. Rao et al. 1998, Vardinon-Friedmann et al. 2000, Zhai et al. 2000 & 2001).

In contrast to the general consensus of synaptic formation described above, the mechanism of rapid synaptogenesis is suggested to use preassembled packets of presynaptic and postsynaptic components to build a synapse (e.g. Rao et al. 1998, Ahmari et al. 2000, Zhai 2001, Schaefer and Nonet 2001; the following chapter, *Figure 4*). Also, certain postsynaptic proteins can form clusters without prior contact with the presynaptic active zone (e.g. O'Brien et al. 1997, Rao et al. 1998).

Many questions concerning synaptogenesis still remain unanswered. Among these questions are: In which developmental stage and in which order contacts are formed, and what other context are needed? What are the precursors of the active zone components? In what form are the synaptic proteins transported to new synapses?

6. Synapse

The following sections of this review combine the present knowledge of presynaptic and postsynaptic structures and events. The focus is on excitatory, chemical synapses.

The concept of synapse developed slowly during the late 19th century after several decades of disputes about the organization of the nervous system. In 1894, English neurophysiologist Charles Sherrington talked about connections between fibres and nerve cells in a speech before the Royal Society of London. He also mentioned that the cells are polarized: "*nerve current always enters by way of the protoplasmic apparatus of the cellular body (*now: dendrite*) and it leaves by the axis cylinder (*now: axon*) which transmits it to a new protoplasmic apparatus.*" Sherrington suggested the name 'syndesm' for the junction between neurons. This name was changed later to 'synapse' (Integrative Action of the Nervous System 1906, described in Elements of Molecular Neurobiology by Smith 1996). Fifty years after Sherrington, Hebb (1949) described neuronal mechanism leading to synaptic modifications, thus a basis for memory and learning: "*When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased.*" Gray proposed in 1959 that synapses consisting of **a presynaptic bouton** in contact with **a dendritic spine** would have an excitatory effect. Unlike the pioneer neurophysiologists, he was able to use a new invention, an electron microscope (Gray 1959). At present, due to the vast complexity and variability of synaptic connections (inhibitory/excitatory), synapses are simply defined as asymmetric junctions composed of **a presynaptic terminal** (a bouton) including neurotransmitter-containing synaptic vesicles, **a synaptic cleft**, and **a postsynaptic apparatus** with neurotransmitter receptors (*Figure 3*; e.g. Garner et al. 2000b).
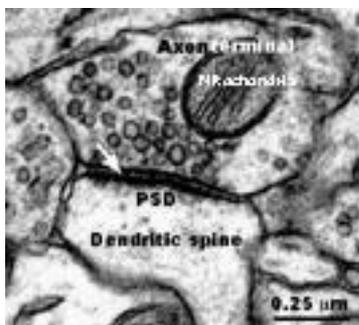
Figure 3. An example of a typical synapse. Synaptic cleft indicated by a white arrow. PSD= postsynaptic density. Copied from www.synapses.bu.edu (EM-picture by J Spacek).

The earlier view of synapses being structurally static is changing; the current leading view of synapses is that they are dynamic in shape, turnover and structural integrity. Recently determined hebbiasome (Husi et al. 2000, more closely on pages 22-23), a large complex of postsynaptic proteins providing the molecular functions required of Hebb synapses, may explain in the future how the diverse set of cellular functions are involved in different patterns of synaptic activity (Grant and O'Dell 2001). Whether these proteins are modulators or effectors of synaptic functions remain to be seen.

6.1 Presynaptic terminal

Presynaptic terminals or boutons of average CNS synapses are only ~1 μm across and are composed of distinct structural and functional compartments. An electron-dense meshwork of cytoskeletal filaments and embedded clusters of synaptic vesicles in association with presynaptic membrane form a specialized region called **the active zone**. The presynaptic cytoskeletal matrix is thought to regulate the mobilization and recycling of synaptic vesicles and enables the active zone to function harmoniously with the receptor apparatuses on the postsynaptic side (e.g. Landis et al. 1988, Garner et al. 2000a, b; Zhai et al. 2000 & 2001).

The presynaptic terminal is a reservoir of three functionally **different pools of ~200 synaptic vesicles** (e.g. Landis et al. 1988, Pieribone et al 1995, Schikorski and Stevens 1997). Those, which reside about 200 nm away from the active zone, form **a reserve pool**. Synapsins form a protein coat around vesicles and anchor the reserve pool vesicles with microfilaments via phosphorylation (e.g. Pieribone et al. 1995, Brodin et al. 1997, Hilfiker et al. 1999). **A proximal pool** of synaptic vesicles is embedded in the cytoskeletal matrix at the neurotransmitter release site. The third **pool of release-ready** synaptic vesicles is docked at the active zone in a fusion-ready state (e.g. Landis et al 1988, Pieribone et al. 1995, Brodin et al. 1997). Approximately 35 vesicles of 200 are thought to undergo recycling in the small CNS terminals (reviewed by Harata et al. 2001).

Structural components of presynaptic terminal
Building blocks for presynaptic terminals include neurotransmitter-containing synaptic vesicles, ion-channel components, and adhesion proteins. They have to be transported by vesicular intermediates, which are also presumed to take part in sorting synaptic components. Cytoskeletal proteins of nerve terminals, such as actin, tubulin, and clathrin, are supposed to be transported by slow-transport mechanisms (e.g. Hirokawa 1989, Hannah et al. 1999). Indication for other types of vesicular transport has also emerged (*Figure 4*).
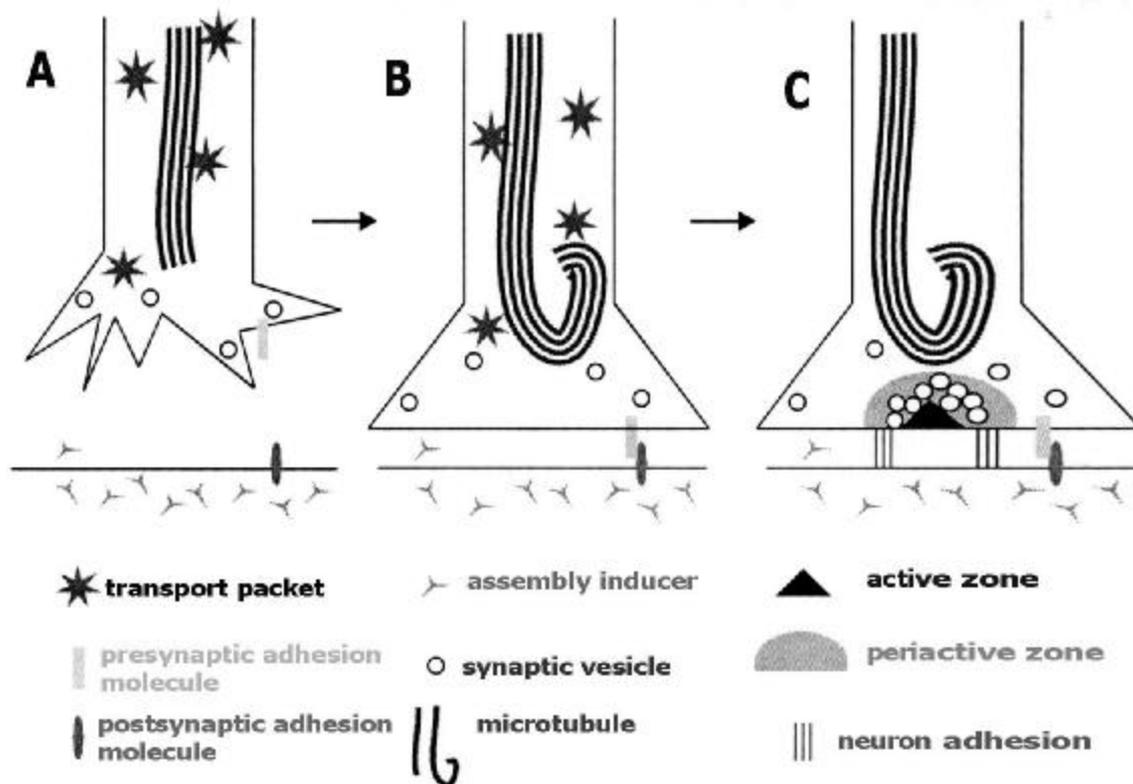
Figure 4. A model of presynaptic assembly (according to Schaefer and Nonet, 2001). A) Transport packets arriving in axonal growth cones bring preassembled complexes of synaptic molecules. B) Interaction of adhesion molecules triggers presynaptic assembly. C) Differentiation of the presynaptic terminal includes appearance of active zone and vesicle clustering. Periactive zone, which surrounds the active zone, includes adhesion proteins and molecules needed for e.g. development.

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Vaughn suggested in 1989 that nerve terminals might obtain building material in part from preformed complexes, such as granulated vesicles. A decade later, two potential components were identified to be involved in active zone assembly. The two proteins, Bassoon and Piccolo, were first detected as structural components of the active zone. Both are large multi-domain scaffold proteins likely to interact with many different proteins (tom Dieck et al. 1998, Fenster et al. 2000). Both of them are expressed at early stages of neuronal differentiation. They arrive at newly forming synapses prior to, or at the same time as the synaptic vesicles induced by neuronal activity (Vardinon-Friedman et al. 2000, Zhai et al. 2000). In immature hippocampal neurons, Piccolo is found to be associated with Golgi-derived granulated vesicles, which are sorted into axons and growth cones as neurons begin to mature (Zhai et al. 2001). As a matter of fact, lots of different types and shapes of vesicles have been shown to accumulate at newly forming synapses (e.g. Kraszewski et al. 1995, Ahmari et al. 2000), but their actual function in the nerve terminals has remained unclear. Recently, Piccolo has been shown to cluster in developing hippocampal neurons with other presynaptic components, such as N-cadherin (adhesion protein), syntaxin, SNAP-25 (synaptosomal-associated protein), and Bassoon (Zhai, 2001). This study showed for the first time that at least some major components of active zone are packed together in transport vesicles and thereby provided new evidence for Vaughn's suggestion.

Certain proteins usually located in the postsynaptic side can be found in presynaptic terminals, depending on the specific protein isoform and neuronal cell type. Among them are members of the membrane-associated guanylate kinase (MAGUK) superfamily, such as SAP-97 and SAP-102 (synapse-associated proteins); PSD-93 and PSD-95/SAP-90 (postsynaptic density proteins) (e.g. Garner et al. 2000b). In hippocampal neurons, SAP-97 and SAP-102 reside both pre- and postsynaptically (e.g. Garner and Kindler 1996, Craven and Brendt 1998), while PSD-93 and PSD-95 occur only in postsynapses (e.g. Cho et al. 1992, El-Husseini et al. 2000). In cerebellar basket cells, however, PSD-95 occurs prominently in presynaptic terminals (Kistner et al 1993, Hunt et al. 1996). PSD-95 localizes also presynaptically in different cells of the retina (Koulen et al. 1998). The major targets of these proteins have been suggested to be interactions with cell-adhesion molecules, cytosolic proteins, and Ca^{2+}-channels (e.g. Cho et al. 1992, Kistner et al 1993, Koulen et al. 1998, Hsueh et al. 1998, Garner et al. 2000b, Aoki et al. 2001). In addition, there are some novel findings that NMDA (N-methyl-D-aspartate) -receptors, which mainly (~99%) reside in the postsynaptic membrane, are also found region-specifically in the presynaptic side of hippocampal synapses (Sequeira et al. 2001). These presynaptic NMDA-receptors may have an autoreceptor role, which could block the release of amino acids from the cytoplasmic pools, hence an opposite function

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to neurotransmitter transporters (Breukel et al. 1999, Sequeira et al. 2001, Aoki et al. 2001).
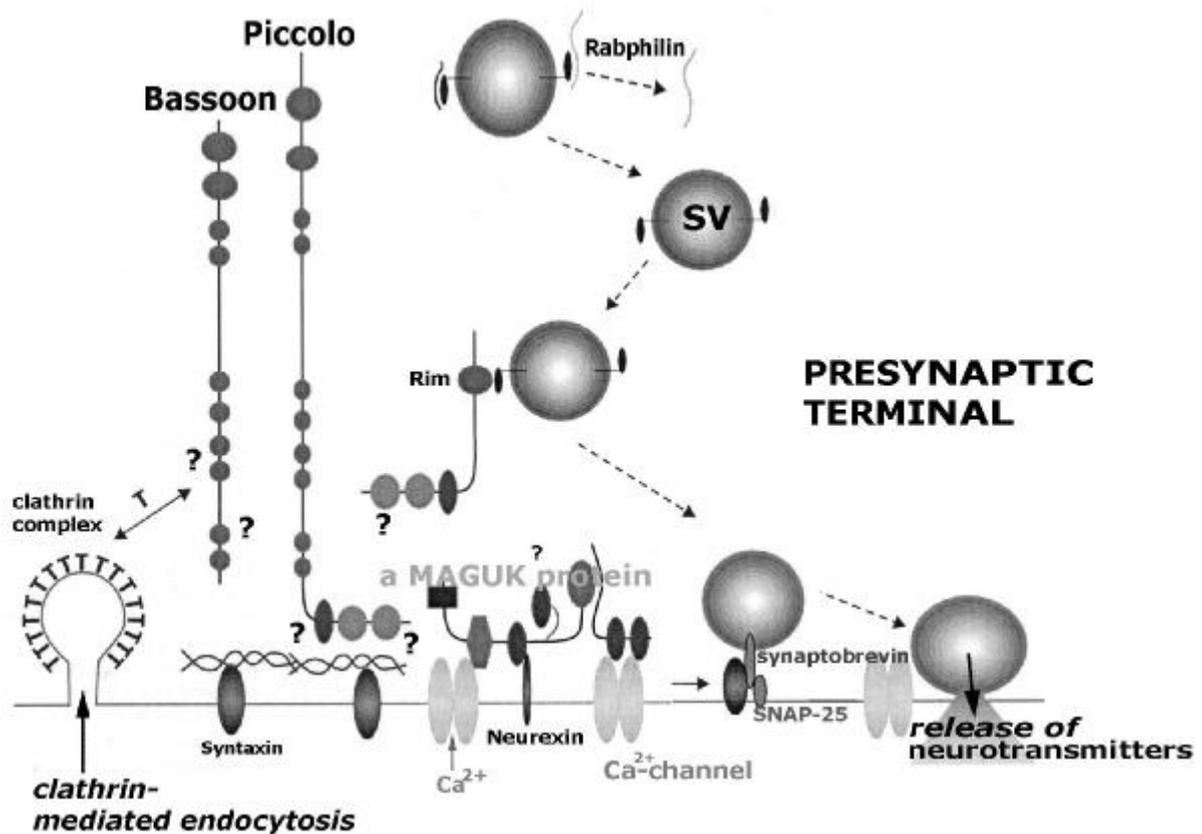
Figure 5. A model of presynaptic molecular structure and synaptic vesicle exocytosis. Modified from Garner et al. 2000a

Synaptic vesicles

Synaptic vesicles in the three functionally different vesicle pools (see previous section) vary in shape and size according to their contents. Small, translucent, and spherical vesicles with a diameter of about 50 nm carry excitatory transmitters such as glutamate. Ellipsoidal, translucent vesicles are believed to contain inhibitory transmitters such as glycine. Larger vesicles with a diameter more than 60 nm often have dense cores and contain catecholamines, whereas even larger (~175 nm) dense-core vesicles contain peptides. Many synaptic vesicles, including those containing transmitters, are thought to carry several soluble proteins, building blocks for synapse formation, and perhaps enzymes needed for final post-translational processing (e.g. Smith 1996, Ahmari et al. 2000, Zhai et al. 2001).

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Formation of functional synaptic vesicles is the first requirement for synaptic transmission to occur. Developmental expression patterns of several synaptic vesicle proteins have been analyzed both at mRNA and protein level. For example, synapsin I mRNA, which encodes a synaptic vesicle -specific 'coat'-protein, is detectable at E12-E14. The mRNA level in various regions of the brain increases parallel to formation of synapse (e.g. Haas and DeGennaro 1988 & 1990, Melloni and DeGennaro 1994). Synaptophysin mRNA, which encodes a transmembrane protein of the synaptic vesicle, is expressed during early embryonic development, while protein levels start to increase later during synapse formation (e.g. Devoto and Barnstable 1989, Leclerc et al. 1989, Bergmann et al. 1991, Marazzi and Buckley 1993, Daly and Ziff 1997). There results are similar to results obtained with two other well-studied synaptic vesicle proteins, synaptotagmin and synaptophysin II (e.g. Lou and Bixby 1995).

Studies of neuronal cultures have shown that synaptic vesicle proteins are present before neurons have differentiated and that an initial level of synaptic vesicle protein expression is modulated during synapse formation (e.g. Fletcher et al. 1991, Daly and Ziff 1997). The modulation mechanism of synaptic protein expression must be complex, since mRNA levels do not always correlate with the protein levels (e.g. Bergmann et al. 1991, Lou and Bixby 1995, Melloni and DeGennaro 1994). In developing embryonic hippocampal neurons, mRNA expression of synapsin, synaptotagmin I, and synaptobrevin is stable. Nevertheless, half-lives of these proteins start to increase progressively as neurons start to develop in culture. In the case of synaptophysin, the amount of protein is upregulated without increase in mRNA level as neurons begin to develop. This predicts an increased rate of translation. Thus, separate regulatory roles apply for certain proteins and perhaps a few key components of synaptic vesicles are developmentally regulated (Daly and Ziff, 1997).

6.2 Postsynaptic apparatus

Dendritic spines

There are various types of synapses with specific characteristics, but the major postsynaptic sites on most principal cells in the cerebral cortex are dendritic spines (e.g. Gray 1959, Peters and Kaiserman-Abramof 1969, Spacek and Hartmann 1983). These structures vary in size, shape, number and distribution in response to brain development and activity (e.g. Spacek et al. 1997). Usually, spines have contacts with one presynaptic terminal (Westrum and Blackstad 1962). Branched dendrites of pyramidal cells, however, can contain thousands of synapses made by axons from about as many neurons. The spine interior consists mainly of spine organelles, mRNA, ribosomes, mitochodria, and either smooth endoplasmic reticulum or spine-apparatus connected to

postsynaptic density. Thus, protein synthesis and postranslational modification of proteins required for a quick spine modification are carried out at the base of the dendritic spine (e.g. Steward et al. 1988, Harris 1994). During development, spines are created from filopodial protrusions (dendritic shafts) that emerge from dendrites and begin to change in size. Some may quickly stabilize to spines and functional synapses, whereas others may retract completely (e.g. Dailey and Smith 1996, Ziv and Smith 1996). Appropriate stimuli are needed to induce the formation of filopodial protrusions and change their form and shape within the immature neurons. The role of filopodia in mature neurons remains to be established. The mature synaptic remodeling however is proposed to occur via actin filament modifications (e.g. reviewed by Lüscher et al. 2000; further discussion in Neosynaptogenesis, page 30).

Postsynaptic density

EM studies in the late 1950s were able to show a thickening structure in the postsynaptic membrane and scientists called it postsynaptic web or postsynaptic density (PSD) (Palay 1958, Gray 1959). Typical PSDs are observed at type 1 glutamatergic excitatory synapses, which have been the focus on the modern day science. Morphologically, PSDs in type 1 synapses may vary from axodendritic synaptic junctions formed on dendritic shafts to nonperforated/perforated continuous/segmented PSDs of various dendritic spine formations (e.g. Peters et al. 1991). Perforated PSDs (which make perforated synapses; *Figure 6*, see also page 31) contain a higher proportion of smooth endoplasmic reticulum. They are also more likely to include a spine apparatus than nonperforated PSDs. Spine apparatuses are organelles that are thought to be involved in membrane synthesis and storage of calcium, which can be released in response to an appropriate stimulus (e.g. Spacek et al. 1997).

Figure 6. A perforated synapse. Arrows point to segmented PSD. Copied from from www.synapses.bu.edu (EM-picture by J Spacek).

In early 1970s, cell-biologists developed a detergent extraction method and used differential centrifugation to isolate and purify PSD structures from brain tissue (e.g. Cotman et al. 1974, Carlin et al. 1980). Primary analyses identified proteins, such as actin and tubulin (Kelly and Cotman 1978), calmodulin (Grab et al. 1979, Carlin et al. 1981), and fodrin (Carlin et al. 1983) as associated with PSDs. Further treatment of PSD fractions, with strong detergents, however, removed proteins like actin and tubulin (Matus and Taff-Jones 1978). Later, actin was found to be in direct contact with the NMDA receptor (reviewed by Ziff 1997). Thus, the initial Triton X-100 extracted structures were suggested to be called synaptic junctions, where pre- and postsynaptic membranes were still in contact. In order to obtain pure PSD-fractions, only harsh detergents, such as sarcosyl, could separate the components keeping these membranes together (e.g. Cotman 1974, Kelly and Cotman 1978, Matus and Taff-Jones 1978).

In 1992, scientists from Mary Kennedy's laboratory recognized one particular protein to be highly enriched in PSD fraction and named it PSD-95. The developmental expression of PSD-95 increased coinciding synaptogenesis and PSD-95 was found at high levels in dendrites (Cho et al. 1992). Later, neurotransmitter receptors, such as the AMPA (DL-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)- and NMDA receptor, and several additional proteins such as kinases, phosphatases, adhesion proteins, and scaffolding proteins have been identified to bind to the receptors or PSDs (reviewed e.g. by Ziff 1997 and Kennedy 2000, Husi et al. 2000). One of the most abundant proteins is CaMKII, a calcium/calmodulin-regulated serine/threonine kinase, which constitutes about 2-5% of the protein in PSD. The role of CaMKII is in neuronal circuit development and regulating synaptic strength (e.g. Kennedy et al. 1983, Lisman and Goldring 1988; reviewed by Söderling 2000, and Cline 2001).

Crucial for the clustering of the proteins in PSD and their synaptic localization is palmitoylation of PSD-95 and other members of the same family. Dual palmitoylation is necessary for anchoring PSD-95 to the postsynaptic membrane (Topinka and Bredt 1998, Craven et al. 1999, El-Husseini et al. 2000). According to a recent polarized trafficking study, however, the N-terminal palmitoylation motif of PSD-95 is insufficient for dendritic targeting. It was suggested that a cytosolic or membrane-associated palmitoyl transferase enzyme, other than the one residing at the *trans*-Golgi network, could recognize the palmitoylation motif (El-Husseini et al. 2001).

Earlier PSD-analyses suggested different functions for the PSD: regulation or aggregation of postsynaptic receptors and stabilization of the synaptic junctions (e.g. Siekevitz, 1985), activation of receptors and signal transduction in response to synaptic activation, (e.g. Kennedy et al. 1983), or storage of information (e.g. Lisman and

Goldring 1988). Several studies of excitatory synapses have suggested that the strength of synaptic transmission could be regulated at the postsynaptic membrane.

Recently, large-scale protein studies have provided evidence to support earlier predictions of the multipurpose role of PSD. More than 500 proteins, identified by proteomic characterization with mass spectrometry and immunoblotting, were localized in PSD. Seventy-five of these proteins bound to the NMDAR/mGluR (metabotropic glutamate receptor)- PSD-95 complex (Husi et al. 2000). This large complex contained 30 earlier identified molecules as well as additional receptor, adaptor, signaling, cytoskeletal, and novel proteins. The authors suggested that Ras-MAPK pathway proteins could form a module within the complex of proteins attached to the NMDA- and mGlu- receptors. Also, cell-adhesion proteins (e.g. N-cadherin, neuroligin, β-neurexin) were proposed to participate in the scaffold holding the synapse and its components together (e.g. Song J-Y et al. 1999, Husi et al. 2000). Thus, for the first time this study showed that physical association of enzymes with receptors explains the involvement of ubiquitous enzymes in specific signaling pathways. In the future, it will be necessary to define the possible organizational variations of signaling machinery in different synapses. In addition, understanding interactions and feedback systems of different signaling pathways in synapses will be a major target (Kennedy 2000).
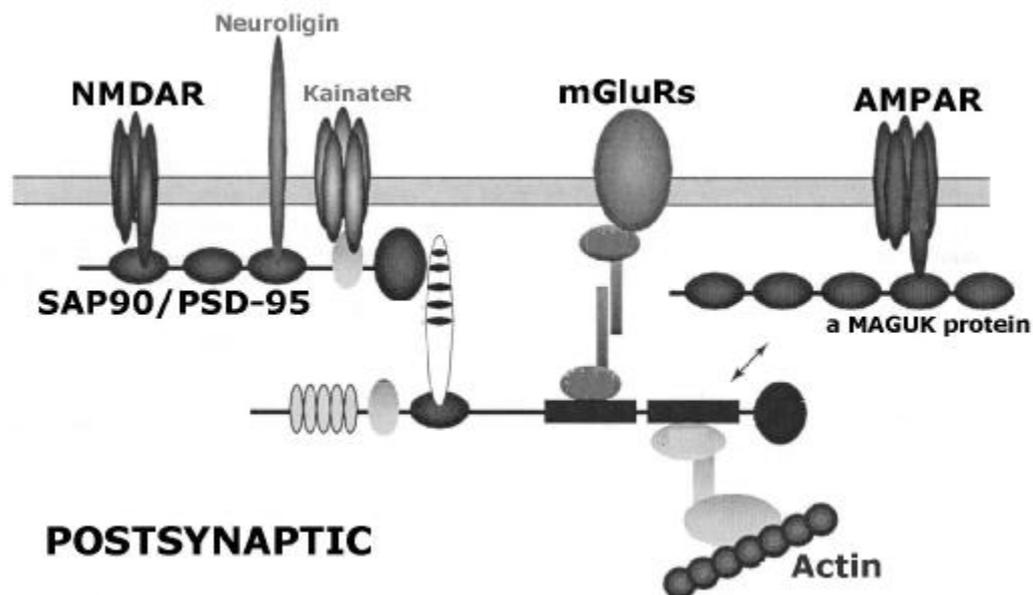
Figure 7. A model of postsynaptic interactions between proteins of postsynaptic density. Modified from Garner et al. 2000b.

7. Synaptic function

Neurons are polarized cells with both axonal and dendritic processes. Electrical impulses enter dendrites, and via the cell soma impulses propagate to axon terminals and covert into chemical signals at synapses. The strength of the impulse and variation of ionic flows due to action potential form the basis of synaptic functions.

7.1 Mechanism of synaptic transmission

An electrical impulse reaching the nerve terminal induces a local influx of Ca^{2+} through voltage-gated calcium-channels localized in the active zone membrane triggers the release of synaptic vesicles from their anchorages (e.g. Dunlap et al. 1995, Wu et al. 1999). vSNARE proteins (e.g. synaptobrevins) of the vesicle membrane mediate the docking and fusion of these released vesicles at the active zone. While tSNARE proteins [SNAP-25 (synaptosomal-associated proteins) and syntaxins] are in charge at the presynaptic membrane (e.g. Südhof 1995, Hanson et al. 1997). Together these aforementioned proteins form the SNARE-complex that is essential for neurosecretion (e.g. Hanson et al. 1997). Synaptotagmin 1, which is located on synaptic vesicles, is a likely Ca^{2+}-sensor for neurotransmission, and the SNARE-proteins possibly interact with synaptotagmins to trigger the fusion (e.g. Schiavo et al. 1995, Südhof and Rizo 1996). Synaptotagmin and SNAP-25 are palmitoylated, and that might affect to the regulation of the synaptic vesicle cycle (Chapman et al. 1996, Hess et al. 1992, Veit et al. 1996). In addition of being a component of the SNARE-complex, synaptobrevin binds to synaptophysin and synaptoporin, the major proteins of synaptic vesicle membrane (Edelmann et al. 1995). Several effector molecules, such as Rim and rabphilin (e.g. Shirataki et al. 1993, Wang et al. 1997), have been identified to take part in synaptic vesicle exocytosis, but their exact role is unknown (see a schematic model in *Figure 5*, page 26)

There are two possible ways to release contents of synaptic vesicles and recycling them: a 'kiss-and-run' –exocytosis followed by rapid endocytosis, or a membrane fusion-type exocytosis - endosytosis (e.g. DeCamilli and Takei 1996, Brodin et al. 1997, Harata et al. 2001). The former allows the speedy reestablishment of the release-ready pool. In the latter case, after membrane fusion and release of neurotransmitters to the synaptic cleft, the synaptic vesicle proteins are recycled via clathrin-mediated endocytosis (e.g. De Camilli and Takei 1996, Brodin et al. 1997). After clathrin -coated vesicles are uncoated, they turn into new synaptic vesicles without any further endosomal step (e.g. Takei et al. 1996, Murthy and Stevens 1998). Some neurotransmitters are transported back into the terminal by specific uptake mechanisms and are reloaded to the new synaptic vesicles. Eventually, these newly recycled vesicles reach the existing pool of

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synaptic vesicles, unless they are transferred to the cell soma for loading with newly translated neurotransmitters (e.g. Betz and Bewick 1992, Kiromi and Kidokoro 1998, Betz and Angelson 1998). Under resting conditions, the synaptic vesicle pool is relatively immobile. After synaptic stimulation, vesicles are drawn to the plasma membrane and cycling continues (e.g. Bezt and Bewick 1992, Bezt and Angleson 1998). This cycling requires a supply of ATP, which is provided by the mitochondria located at the presynaptic terminal (see *Figure 3*, page 21; e.g. Brodin et al. 1999).

7.2 Changes in synaptic activity: Pruning and Neosynaptogenesis

Modifications of synaptic strength vary during and beyond CNS development due to changes in synaptic activities that can modulate the composition of postsynaptic membranes and dendritic spine structures. These modulations may strengthen existing synapses, or form new synaptic interactions. Also, depending on the nature of synaptic activity, they may shut down some synaptic connections.

As mentioned earlier, Ca^{2+} and Ca^{2+}-channels play a vital role in the release of neurotransmitters from nerve terminals. Arrival of an impulse opens the Ca^{2+}- channels by depolarizing the membrane of the excitatory terminal. Influx of Ca^{2+}-ions triggers a chain of events that release synaptic vesicle contents into the synaptic cleft. Calcium is also involved in long-term potentiation (LTP) processes, acting in the postsynaptic membrane via the NMDA-receptor. In LTP, brief high frequency electrical stimulation of the neural pathway strengthens synapses, and it has a long-lasting effect. The obverse of LTP is long-term depression (LTD), where the stimulus is weak for a long period of time. LTP and LTD provide means by which certain neuronal pathways can become differentiated from the zillions of others that exist in the mammalian brain. Particularly, since it is the postsynaptic cell, which is affected by LTP (or LTD), not the presynaptic ending. Several LTP/LTD-studies have led to a model proposition that intracellular Ca^{2+} changes can have numerous effects on spines (e.g. Harris and Kater 1994, Segal et al. 2000). Minimal synaptic activation is required for spine maintenance, while even slight increases in Ca^{2+} can cause growth and formation of new spines (neosynaptogenesis). Extensive increases instead cause spine retraction (pruning).

Studies in the mid-nineties showed increases in spine intensity following LTP (e.g. Geinisman et al. 1996, Trommald et al. 1996). Later, it was also observed that the amount of multiple-spine synapses, where two adjacent spines arise from the same dendrite and contact a single presynaptic terminal, increased after LTP (Toni et al. 1999). Whether it is the *de novo* spine formation or PSD-splitting that causes changes in spine density remains to be confirmed. Recently published laser-scanning microscopy studies of activity dependent growth of new filopodia or spine formations from a

dendritic shaft or from existing spines bring evidence of *de novo* formation (e.g. Maletic-Savatic et al. 1999, Engert et al. 1999). There is still the enigma, however, of pre-existing PSD clusters (perforated synapses, *Figure 6* in page 28) which could be transformed into independent synapses by splitting or budding (Toni et al. 1999).

If neosynaptogenesis can be investigated by inducing LTP, LTD could be used for studying synaptic pruning. Although little is known about the LTD-induced changes in synapses, numerous studies have predicted that the opposite events are involved in LTD compared to LTP. These would include loss of certain receptors, shrinkage of PSDs and complete disappearance of dendritic spines and corresponding presynaptic boutons (e.g. Lüscher et al. 2000).

Synaptic activity altered by transient ischemia and experimental epilepsy also causes changes in PSD structures and compositions (e.g. Hu et al. 1998, Martone et al. 1999, Wyneken et al. 2001). Particularly in experimental epilepsy, neurons have been shown to lose spines (Drakew et al. 1996). On the other hand, even brief seizure episodes have been reported to induce neosynaptogenesis and synaptic reorganization (e.g. Ben-Ari and Represa 1990, Represa and Ben-Ari 1992 & 1997, Perez et al. 1996, Esclapez et al. 1999).

An EM study of cerebral biopsy specimen from a CLN2 patient in a moderately advanced stage showed that most of the synapses were still normal, although some abnormally elongated spines were observed. Surprisingly, Williams et al. found loss of type II synapses, which are known to be inhibitory (e.g. Carlin et al. 1980), thus leaving type I excitatory synapses to dominate the signaling in the affected brain (Williams et al. 1977). Whether the same applies to CLN1 or other NCL-types remains to be investigated.

8. Neuropathological model: Experimental epilepsy

Kainic acid (KA, kainate) -induced experimental epilepsy is probably the most popular *in vivo* model of neuronal excitotoxicity. KA, an analog of excitatory amino acid glutamate, is one of the best-studied excitotoxins, usage of which leads to selective and delayed neurodegeneration similar to human temporal epilepsy.

In 1953, Dr Takemoto extracted KA from seaweed *Dignea simplex* (in Japanese, Makuri or *Kaininso)*, one of the red algae that grows e.g. in the Indian Ocean, the Red Sea and the East China Sea. It used to be an ancient remedy for intestinal parasites. KA

paralyzed the worms. After testing with rats and crayfish, Japanese scientists found out that KA had strong excitatory activity, as expected due to the chemical similarities between glutamate and KA (McGeer et al. 1978).

Studies in the 1970s showed that local or distal injections of KA into adult rats evoked seizures, which propagate from the hippocampal regions to the entorhinal cortex and other limbic structures (e.g. McGeer et al. 1978, Nadler 1981, Ben-Ari 1985). The most responsive neurons to KA in the rat brain are CA3 pyramidal neurons of the hippocampus. They are very sensitive to hyperactivity *per se*, and degenerate following KA-induced seizures. Thus, a low concentration (nanomolar range) of distally injected KA, which can reach the brain, is enough to generate seizures and brain damage including the loss of CA3 neurons (e.g. Ben-Ari 1985).

Appearance of seizure-induced lesions depends on the maturity of the brain. The neuronal damage is observable only after the granule cells and mossy fiber synapses become functional at the third postnatal week (e.g. Tremblay et al. 1984, Ben-Ari 1985, Routbort 1999). The mossy fiber synapses connecting granule cell axons (mossy fibers) to CA3 pyramidal neurons are needed to bring KA to postsynaptic high-affinity receptors. Therefore, CA3 pyramidal neurons, which are rich in high-affinity KA-receptors, are activated first. The synchronized glutamatergic currents formed in CA3 neurons propagate to the CA1 region, which is the major output gate from the hippocampus to other limbic structures (e.g. reviewed by Ben-Ari and Cossart 2000). In the chronic epileptic state of rats, pyramidal neurons of the CA1 region are able to grow novel axonal branches and form new excitatory pathways with new synaptic connections that enable hyperexcitation to continue and spread to other regions of the brain (e.g. Perez et al. 1996, Esclapez et al. 1999).

Genetic response to KA-induced hyperexcitation is vast and varied. At mRNA level alone, more than 300 plasticity-related genes are upregulated. These include several neurotrophic factors, synaptic proteins, and proteases marking the early neuronal response. Usually the early-responding genes return to normal level within approximately 4 days after KA-induced seizures. Down-regulation of about 50 plasticity-related genes has also been reported (Hevroni et al. 1998, Zagulska-Szymczak et al. 2001). At protein level, those representing neuronal loss (neurofilaments, α-internexin), disruption of cytoskeleton (tubulin), mitochondrial derangement (mitochondrial enzymes), and heat-shock proteins (HSP27) have been shown to change in the rat brain 1 week after KA-treatment (e.g. Krapfenbauer et al. 2001a). Also, the levels of sparse cytosolic proteins of the rat brain, such as annexin VII (apoptotic marker), SP-22 (antioxidant protein, protects enzymes from oxidative damage), and HOP (chaperone

cofactor) have decreased. The protein levels of heparin-binding protein p30, which is involved in neural differentiation, have increased due to KA, indicating an induction of repair mechanism. Thus, apoptosis, heat-shock system, antioxidant response, and repair mechanisms are activated and continue functioning 1 week after KA-treatment (Krapfenbauer et al. 2001b).

KA-induced hyperactivation of synapses can also modify the structure of PSD. Six hours after KA-treatment, the total protein content of PSD has already increased. The amount of glutamate receptors and associates, kinases, and other synaptic signaling proteins have also increased during that time. In contrast, components of the PSD-structure itself showed diminished protein levels (Wyneken et al. 2001).

AIMS OF THE STUDY

To study developmental expression of PPT1 in rats.

To compare expression patterns of PPT1 with other lysosomal proteins involved in NCLs.

To determine the subcellular localization of PPT1 in neurons.

To examine the effect of hyperexcitation in PPT1 expression and localization.

MATERIAL AND METHODS

1. Animals and tissue material

Two independent developmental series (E11-adult) of Wistar rat brain tissue and one series of Wistar rat liver (E16- adult), spleen (E18-adult), and eyes (E18-adult) were collected and analyzed. All tissue samples were frozen immediately on dry ice – ethanol and stored at –80 °C. Hippocampi of P10-13 Sprague-Dawley rats were used in the organotypic slice cultures. For hyperexcitation studies, 2 months of age adult Sprague-Dawley rats weighing 320-420 g (n= 38) and P15 rat pups weighing 30-32 g (n= 8) were analyzed. Animals were treated according to the ethical guidelines of the European convention approved in Strasbourg in 1986. The animal studies were approved by the Institutional Animal Care and Use Committee of the University of Helsinki (developmental studies; licensed to Jaana Vesterinen) and Åbo Akademi University (excitotoxicity studies; licensed to Pertti Panula).

2. Kainic acid treatment of rats

Kainic acid (KA, 2-carboxy-4- (1-methylethenyl)-3-pyrrolidineacetic acid, Tocris Cookson Inc., Ballwin, Missouri) dissolved in 0.9% sodium chloride (NaCl) was used to induce status epilepticus in adult (n= 30) and P15 (n= 4) rats. P15 rats were injected subcutaneously with 4 mg/kg KA and adult rats with 12 mg/kg. Control adult (n= 8) and P15 (n= 4) rats were treated with corresponding amounts of 0.9% NaCl. Behavioral changes of rats were observed for at least 3 h after KA-injection. During the first 20-30 min, animals had staring spells, followed by head nodding and numerous periods of wet-dog shakes for approximately 30 min. One hour after KA-injection, there appeared recurrent limbic motor seizures, such as facial movements, forepaw tremor (~swimming movements), rearing, and loss of postural control. Then the seizures became prolonged and more complex. Usually, a full status epilepticus occurred within 2 h after KA-injection (Ben-Ari 1985). All pups treated were returned to the lactating mother, which accepted them without hesitation.

3. Histochemistry

Perfusion. Adult rats were anesthetized with sodium pentobarbital at time intervals of 6h, 24 h, 72 h, 1 week, and 1 month after KA. P15 rats were decapitated at 24 h or 72 h

after KA. The brains were perfused intracardially with saline (200 ml), followed by ice-cold 4% paraformaldehyde (250 ml, PFA) in 0.1 M phosphate buffer (PB), pH 7.4. The brains were removed, postfixed for 2 h in the same fixative at 4°C, and cryoprotected in 20% sucrose - 0.1 M PB overnight at 4°C. The brains were covered with embedding matrix (Lipshaw, Pittsburg, Pennsylvania) at −20°C and stored frozen. Serial coronal sections (20 μm) were cut on a cryotome, mounted on microscope slides and dried for 2 h before storing at −20°C.

Nissl staining. Rehydrated cryosections were stained with toluidine blue (0.1% in distilled water) to verify the extent of cell damage in the brains of KA- and control rats. After destaining with water, the sections were allowed to dry overnight.

4. Reverse transcription polymerase chain reaction, RT-PCR

Total RNA was extracted using RNeasy kit (Qiagen). All samples were DNase-treated for 20 min at 37°C and the RNA concentration was measured before subjected to RT-PCR reaction. An equal amount of RNA (100 ng/ 10 μl reaction) was analyzed for PPT1 mRNA, as well as for prosaposin, TPP-I, and cathepsin D mRNA by RT-PCR using EZ r*Tth* RNA PCR kit (Perkin-Elmer). β-actin mRNA was always analyzed as a control in each set of experiments. PPT, β-actin, prosaposin, TPP-I, and cathepsin D primers were as described. All primers were designed and selected so that reaction conditions could be kept the same in each case. After optimizing the RNA template and primer concentrations, as well as the number of PCR-cycles, the quantitative changes of reactions could be kept in a linear range. Reverse transcription was at 60°C for 30 min. The resulting cDNA was amplified by PCR, which included: denaturation (5 min, 95°C), 27-32 cycles of denaturation (1 min, 95°C), annealing, and elongation (1 min, 55°C). Final extension (7 min, 55°C) was followed by immediate analysis of PCR products on TBE (89 mM Tris-base, 89 mM Boric acid, 2 mM EDTA) – agarose gels.

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5. Protein studies

Antibodies used in immunohistochemical staining, immunofluorescense, and Western blot analysis are listed below.

Antibodies	Reference	Study
Primary polyclonal antibodies:		
Rabbit anti-PPT1	Prof. Sandra Hofmann*	I-IV
Rabbit anti-PPT1 (trunc.)	Suopanki et al. 1999	I-IV
Rabbit anti-TPP-I	Prof. Eiki Kominami**	III
Commercial monoclonal antibodies:		
Mouse anti-cathepsin D	Biodesign International	III
Mouse anti-GAP-43	Transduction Laboratories	I, III
Mouse anti-LAMP-2	Hybridoma Bank	IV
Mouse anti-NMDAR2B	Transduction Laboratories	IV
Mouse anti-SNAP-25	Transduction Laboratories	unpublished
Mouse anti-PSD-95	Transduction Laboratories	IV
Goat anti-Rab7	Santa Cruz Biotechnologies	IV
Mouse anti-synapsin IIa	Transduction Laboratories	IV
Mouse anti-synaptophysin	Biomeda	IV
Commercial Secondary antibodies:		
Horseradish peroxidase conjugated goat anti-rabbit	Bio-Rad Laboratories	I, III, IV
Horseradish peroxidase conjugated goat anti-mouse	Bio-Rad Laboratories	I, III, IV
Alexa Fluor 488 donkey anti-goat IgG conjugate	Molecular Probes	IV
Alexa Fluor 546 goat anti-rabbit IgG conjugate	Molecular Probes	IV
Alexa Fluor 488 goat anti-mouse IgG conjugate	Molecular Probes	IV

* Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas,
** Department of Biochemistry, Juntendo University School of Medicine, Tokyo, Japan

Immunohistochemistry. Paraffin sections (5 μm) were pretreated with 4% pepsin (w/v) in 0.37% HCl for 40 min at 37°C. Frozen coronal brain slices (20 μm) were thawed for 1 h, rehydrated in PBS, pH 7.4, and pretreated with 4% pepsin (w/v) in 0.37% HCl for 5 min at 37°C. Both paraffin and cryosections were incubated in methanol - 0.3% H_2O_2 for 30 min at RT to block endogenous peroxidase activity. After two washes in PBS - 0.25% Triton (PBST), the sections were incubated with the primary antiserum followed by biotinylated secondary antiserum in combination with avidin - biotinylated horseradish peroxidase complex in PBST, according to the manufacturer's instructions (Elite Vectastain ABC kit, Vector Laboratories). The paraffin sections were stained with 3,3-diaminobenzidine tetrahydrochloride dihydrate (DAB, Sigma) and the cryosections were stained with nickel enhanced DAB for 5 – 10 min at RT. Rinsing with Tris buffer and distilled water stopped the reaction and the slices were allowed to dry overnight before

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being mounted. For negative control, the slices were treated as above, except either omitting primary antiserum or incubating with preimmune serum.

Immunofluorescense. Rehydrated coronal brain slices were pretreated with 50 mM NH_4Cl for 10 min and blocked with 5% BSA in PBST. They were incubated simultaneously with rabbit-anti PPT1 (1:500) and one of the following monoclonal antibodies: LAMP-2 (1:500), NMDAR2B (1:50), Rab7 (1:500), SNAP-25 (1:200), or synapsin IIa (1:500). Overnight incubation at 4°C was followed by washes in PBST and incubation with appropriate Alexa Fluor conjugates (1:500) for 1 h at RT. The double fluorescence was viewed with a Zeiss laser confocal scanning microscope (LSM 510) set at excitation wavelengths 488 nm (FITC) and 546 nm (TRITC) and emission at 505-550 nm (FITC) and >560 nm (TRITC).

Protein extraction from hippocampal sections. Hippocampi of six 20 μm-thick KA and control brain slices, which were cut for immunohistochemical purposes, were scraped from microscope slides and pooled. Proteins were extracted (according to Ikeda et al. 1998) in 2% SDS - RIPA buffer (1% Triton X-100 –1% DOC -10 mM Tris-HCl, pH 8.0 - 150 mM NaCl) by incubating first at 80°C for 20 min and then at 60°C for 2 h. The tissue lysates were centrifuged at 15 000 x g at 4°C for 20 min. Protein concentrations were measured according to Bradford (1976) and equal aliquots (20 μg) were analyzed by Western blots. Extractions were repeated three times.

Gel electrophoresis and Western blotting. Tissue samples from developmental series, as well as human brain and liver samples, were homogenized 0.2 g/ ml in Tris buffered saline (TBS) containing protease inhibitors (Complete, Boehringer Mannheim) and incubated for 10 min on ice. After centrifugation at 1 000 *x g* for 5 min at 4°C, pellets were resuspended in TBS and recentrifuged. Then the combined supernatants were subjected to ultracentrifugation at 200 000 *x g* for 20 min and the obtained supernatant was analyzed.

Protein concentrations were determined (Bradford 1976), samples were run on acrylamide gels (Laemmli 1970) and Western blotting was performed as described earlier (Towbin et al. 1979) except that the immunoreactive bands were visualized with an ECL-system (Enhanced chemiluminescense, Amersham Pharmacia, Uppsala, Sweden) according to manufacturer's instructions. Primary antibodies were cathepsin D (1:1000), GAP-43 (1:1000), NMDAR2B (1:250), PPT1 (1:3000), PSD-95 (1:250), SNAP-25 (1:1000), synaptophysin (1:500), synapsin IIa (1:5000), and TPP-I (1:300). Horseradish peroxidase-conjugated goat anti-mouse (1:1000) and goat anti-rabbit (1:3000) were used as secondary antibodies.

6. Subcellular fractionation

Brain homogenates were fractionated according to previously published protocols with minor adjustments mainly concerning sample volumes. N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (Hepes) was used as a buffer instead of $NaHCO_3$. All buffers mentioned below contained protease inhibitors (Complete, Boehringer Mannheim, Germany) and were stored in a cold room. Samples were processed and kept on ice throughout the procedures and all centrifugations were done at 4 °C. Purity of the preparations was checked by electron microscopy. Isolations were repeated three times.

Isolation of synaptic junctions. The method for synaptic junction preparation was modified and shortened from the initial procedure (Cohen and Siekevitz 1978, Carlin et al. 1980, Cho et al. 1992). Briefly, two cerebral cortices of control rats were homogenized by 10 strokes with a Teflon-glass homogenizer, using 1 g (wet tissue weight)/ 4 ml of 320 mM sucrose - 1 mM $MgCl_2$ - 0.5 mM $CaCl_2$ – 4 mM Hepes, pH 7.3. The homogenate was centrifuged first at 1 500 *x g* for 10 min to pellet nuclei, mitochondria, and cell debris. Then the supernatant was centrifuged at 14 000 *x g* for 15 min and the resulting pellet, containing synaptosomes, was resuspended in 320 mM sucrose – 4 mM Hepes, pH 7.3, using 2.4 ml / 1 g starting tissue. The resuspended material (3 ml) was layered on a discontinuous sucrose density gradient consisting of 3 ml each of 0.85 M, 1.0 M, and 1.2 M sucrose solution in 4 mM Hepes. Gradients were run for 2 h at 80 000 *x g*, using the SW 40Ti rotor (Beckman). Synaptosomes were collected from the border of 1.0 M/ 1.2 M sucrose and diluted in ice-cold 320 mM sucrose – 4 mM Hepes, pH 7.3 containing 0.5% Triton X-100. After 15 min incubation on ice, the suspension was centrifuged at 32 000 *x g* for 20 min to obtain the first synaptic junction pellet. The pellet was reextracted with Triton and centrifuged at 200 000 *x g* for 1 h to obtain the final fraction of synaptic junctions. Samples were analyzed by gel electrophoresis and Western blotting.

Isolation of synaptic vesicles. Synaptic vesicles were prepared as described (Jo et al. 1999). Two cerebral cortices were pooled and homogenized in 320 mM sucrose – 4 mM Hepes, pH 7.3 as described above. Synaptosomes were isolated by centrifuging first at 2 000 *x g* for 10 min and then at 10 000 *x g* for 15 min. After washing, synaptosomes were centrifuged at 12 000 *x g* for 10 min. The pellet was resuspended in 1 ml of the homogenization buffer, diluted 1:10 in cold MilliQ water, and incubated on ice for 15 min to lyse subcellular organelles. Centrifugation at 30 000 *x g* for 20 min resulted in a heavy membrane pellet. A synaptosomal vesicle pellet and a synaptosomal cytosolic supernatant were separated by centrifugation of the supernatant at 250 000 *x g* for 2 h. The synaptosomal vesicle pellet was resuspended in 40 mM Tris-HCl, pH 8.0 for

measuring the protein concentration and for further analysis by SDS-PAGE and Western blotting.

Preparing crude pre- and postsynaptic fractions of hippocampus. Fractionation of hippocampi was performed as described (Cho et al. 1992, Smalla et al. 2000). Hippocampi of two KA-treated rats with similar seizure history or hippocampi of control rats were pooled and homogenized in ice-cold 320 mM sucrose - 12 mM Tris-HCl, pH 8.0. After cell debris and nuclei were removed (1 400 x g, 15 min), the supernatant was centrifuged at 13 000 x g for 20 min to spin down different vesicles and synaptosomes. This pellet was resuspended in the above described buffer containing 1% TX-100. Resuspension was incubated on ice for 1h and centrifuged at 100 000 x g for 1 h. The pellet was washed by rehomogenizing and centrifuging at 100 000 x g for 15 min. Half the pellet thus obtained and containing crude synaptic junctions was treated with 3.0% sarcosyl for 15 min at RT and centrifuged at 100 000 x g for 15 min to separate pre- and postsynaptic membranes (Cotman et al. 1974). Aliquots of the fractions representing equal amount of hippocampal tissue were analyzed by gel electrophoresis and Western blotting.

7. Hippocampal slice culture and KA induction

Hippocampal slice cultures were essentially prepared as described earlier (Stoppini et al. 1991, Lahtinen et al. 2001). Dissected hippocampi of P10-13 were cut transversely (400 μm) using a McIlwain tissue chopper. Then the slices were placed in a bicarbonate-buffered Ringer solution (124 mM NaCl - 3 mM $CaCl_2$, 23 mM $NaHCO_3$ - 1.1 mM NaH_2PO_4 - 2 mM $MgSO_4$ - 10 mM glucose, equilibrated with 95% O_2 – 5% CO_2 to pH 7.4) for 1 h at 26°C. Three to four slices, which contained the complete dentate gyrus and hippocampus proper, were selected per well. They were grown on Millicell-CM membranes (Millipore) with 1 ml growth medium [50% minimal essential medium supplemented with Hepes and bicarbonate (Gibco) - 25% Hanks' balanced salt solution (Gibco) - 25% horse serum - 4 mM L-glutamine - 6.5 mg/ml glucose 25 μg/ml chloramphenicol (Sigma)]. The slice cultures were grown at 35°C under 5% CO_2 in the air usually for 12-15 days until treatment with neurotoxin. Experiments included various incubations with kainic acid (6 μM, 60 μM or 600 μM) or glutamate (10 mM) for several periods of time (from 5 – 30 min) depending on the dose. After brief washes with fresh medium, the slices were allowed to recover for 2 h, 20 h, 48 h, 75 h, or 1 week. Each time point always contained one set of untreated controls. The extent and time course of neuronal damage was monitored by adding propidium iodide (PI, Sigma) 1-2 μg /ml, for 30 min to the medium. PI penetrates damaged cell membrane, thus allowing

visualization of dead cells by inverted fluorescent microscope (Leica DMIL). After the incubation periods, the slices were collected for mRNA and protein analysis: 2 slices /20 μl homogenization buffer /analysis. RT-PCR and Western were performed as described earlier.

RESULTS

1. Developmental expression of PPT1

mRNA levels of PPT1 were analyzed from neuronal and nonneuronal tissues in order to compare the possible differences in transcription patterns during rat development. A previous study showed earlier (Camp et al. 1994) that the expression of PPT1 gene in adult rats is higher in the brain and spleen than in the heart, liver, or kidney. In the present study, the spleen and liver represented the nonneuronal tissues. The cerebrum, the cerebellum, and the eyes were chosen for the neuronal tissues. The same developmental series were used in both mRNA and protein analysis.

1.2 Neuronal tissue vs nonneuronal tissue

mRNA expression. RT-PCR results showed that the expression of the PPT1 gene was much higher in the rat brain, eye and spleen than in the liver. The developmental expression of PPT1 changed dramatically in the rat neuronal tissue, but not in the nonneuronal tissue (*Figure 8*). In the **cerebrum**, the expression was relatively stable throughout the embryonic development. After birth, PPT1 mRNA seemed to have a two-phased expression: 1) a moderate increase until P6, followed by a dramatic drop at P6 - P9; 2) a considerable, progressive increase from P9 to early adulthood. In the **cerebellum**, the mRNA level of PPT1 had more variation than in the cerebrum. Peaks at P1, P6, and P12 possibly reflected neuronal rearrangements or differentiation of neuronal populations occurring throughout the postnatal period until P30. Neuronal migration in the cerebellum finishes around P30, while neuronal migration in the cerebrum has already finished by the first postnatal week (Jacobson, 1991). mRNA expression in the **eyes** was stable during late embryonic - early postnatal days and reached maximum at P9. After P9, the PPT1 expression dropped. But simultaneously with the programmed retinal cell death and eye opening (P14-P18) PPT1 expression increased again and reached a more steady level by early adulthood.
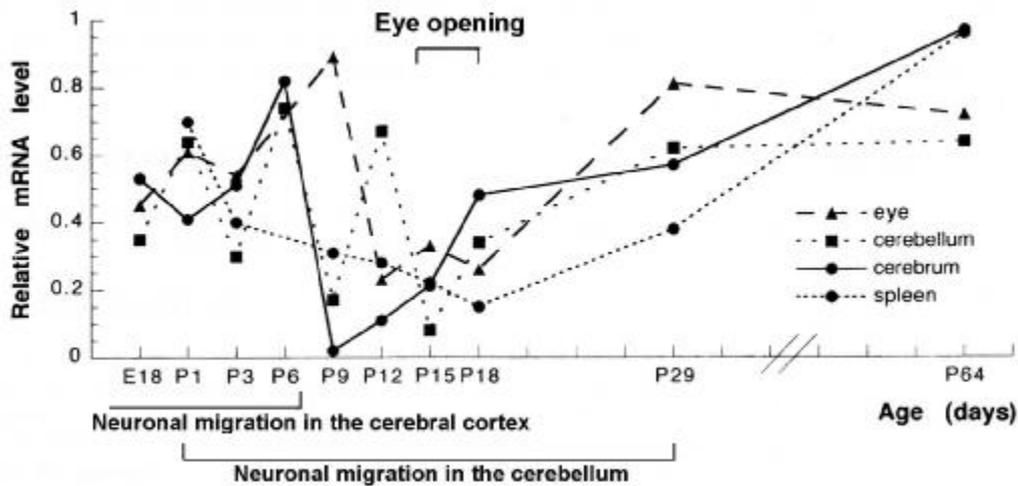
Figure 8. Relative expression levels of PPT1 mRNA in neuronal and nonneuronal tissues. Suopanki et al. 1999, II.

Compared to the developmentally regulated expression in the brain, PPT1 mRNA expression in the **liver and spleen** was considerably stable without any peaks throughout the development. In the liver, the PPT1 mRNA expression slowly decreased during the maturation, in the spleen it started to increase after P18.

Protein expression. The earliest detectable PPT1 protein level **in the brain** was seen at E16 as a 37/39 kDa band (*Figure 9*, left panel, small arrow). After birth, PPT1 protein was produced in a linearly increasing manner and reached the highest concentration in early adulthood. A higher molecular weight immunoreactive band around 70 kDa was detected first at P12 (as single band at P1, P6) and its intensity increased in parallel with the PPT1 doublet towards early adulthood (*Figure 9*, ✶on the left panel). This band was only recognized with one PPT1 antiserum, which did not detect it from the human brain (a big arrow in *Figure 9*). Whether it represented a species-specific dimer of PPT1 remains to be analyzed.
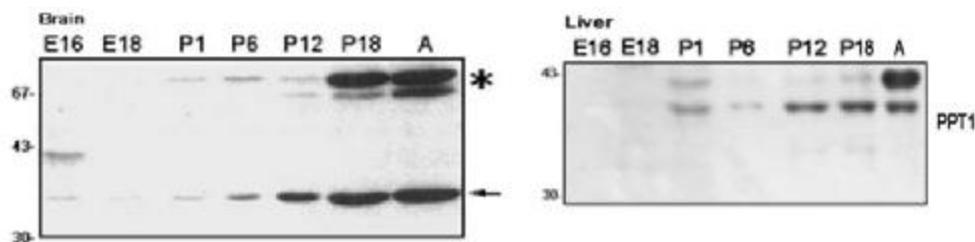
Figure 9. Western analysis of developing rat brain (left, Suopanki et al. 1999, I) and liver (right) tissues stained with anti PPT1 antiserum. A small arrow on the left panel points to 37/39 kDa glycosylated form of PPT1 and ✶ to yet unidentified band of ~70 kDa.

In the liver, PPT1 protein was expressed only after birth, increased during maturation, and was relatively stable after the second postnatal week. Our PPT1 antiserum recognized two bands in the liver tissue: a lower band corresponding to 37/39 kDa glycosylated form of PPT1 and a higher band of approximately 43 kDa (indicated by a big arrow in *Figure 9*, unpublished). The 43 kDa band was also detectable in P1, but became very prominent only at early adulthood. This band was detected in the normal human liver as well as in the INCL liver homogenate (*Figure 10*). The INCL liver had about 1/10 of the amount of the 43 kDa band compared to the normal human liver (by densitometric scanning). At the moment we do not know the identity of this protein. In addition to the 37/39 kDa PPT1 band detected in the brain and liver homogenates, our PPT1-antiserum recognized a heavily smeared unidentified band in the INCL-brain homogenate (unpublished, *Figure 10*). Sequencing attempts were not successful, perhaps due to the blocked N-terminus.
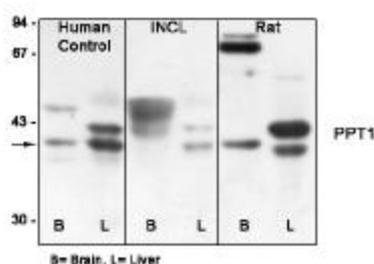
Figure 10. Western analysis of brain and liver homogenates from human control, INCL and rat using anti PPT1 antiserum. A small arrow in the left panel (human control) points to 37/39 kDa glycosylated form of PPT1. On the right panel a big arrow points to ~70 kDa yet unidentified band. Unpublished.

1.2 Developmental expression of TPP-I and cathepsin D

Developmental expression patterns of TPP-I and cathepsin D, the two other lysosomal proteins involved in NCL-diseases, were different compared to that of PPT1. Despite a peak in TPP-I expression at P15, cathepsin D and TPP-I mRNA production continued at relatively constant level throughout the brain development. Again, mRNA production in the spleen, the nonneuronal control, had no major variation during development.

At the protein level, TPP-I had similar expression to PPT1, while cathepsin D expression differed clearly from these two lysosomal proteins. In the brain, TPP-I precursor (~67 kDa) was already detected at E16. The precursor levels increased after birth and reached a maximum at P12. In contrast, the mature form (~46 kDa) was undetectable until P12, thus suggesting an effective processing of the precursor into an enzymatically active mature form after the first postnatal week. At P12 and after, two smaller protein bands were also recognized by anti-TPP-I. Whether these bands represented cleavage products or alternatively glycosylated forms of the mature TPP-I remained to be analyzed. Cathepsin D had two forms: a 43 kDa single chain processed form, which already appeared at E16 and is the major active form in the rat brain (Conner 1998);

and a 30 kDa heavy-chain form, produced only at low levels in rat lysosomes (Conner 1998), was detected from P6 onward.

2. Localization of PPT1 in neurons

Initial immunohistochemical analysis of PPT1 in the adult rat brain showed a granular staining pattern in all types of CNS neurons. The immunoreactivity was located mainly in the cell soma. Certain neuronal populations, such as hippocampal pyramidal neurons and pyramidal neurons at III/IV cortical layer, had a more intense staining. In glial cells, the PPT1 immunoreactivity was faint or non-existing.

2.1 Principal vs nonprincipal neurons of the hippocampus

PPT1 localization in hippocampal neurons was investigated more closely by double immunofluorescence microscopy. Surprisingly, PPT1 localized in various types of neuronal vesicles and the localization varied in different neuronal populations.

Distribution of PPT1 in principal cells (pyramidal neurons) compared to nonprincipal cells (interneurons) of the hippocampus was clearly different. In principal cells, PPT1 was found partially in lysosomes, endosomes, and synaptic vesicles, although PPT1 colocalized best with ionotropic glutamate receptor 2B subunit (NMDAR2B) marking the synaptic membranes. In contrast, the best colocalization of PPT1 in nonprincipal cells was with late-endosomal marker (Rab7) as well as with synaptic marker (synapsin IIa). Assumably, different activities/ metabolic states of these neuronal populations or even individual neurons affected the distribution of PPT1.

2.2 PPT1 in synapse

The vesicular distribution of PPT1 led to further investigations of freshly isolated synapses. By synaptic fractionation, different compartments (synaptic vesicles, synaptic junctions in *Figure 11*) were separated for Western analysis (*Figure 12*).
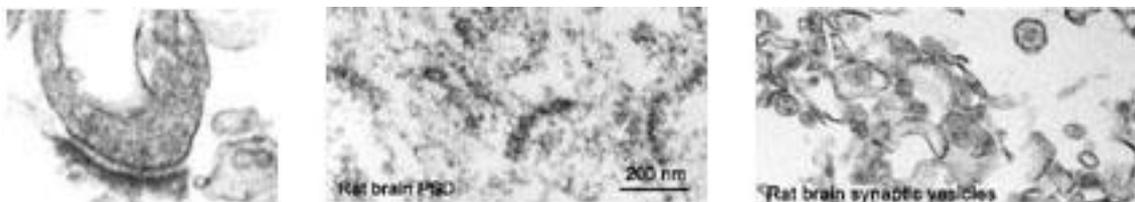
Figure 11. EM-pictures of isolated fractions from adult rat cerebral cortex. Left panel: PSD fraction, middle: synaptic junction (j), right panel: synaptic vesicles (v). [Partanen, Suopanki, Tyynelä, unpublished results].

PSD-95 and synaptophysin were monitored as control protein marking the separation of junctions and vesicles. **In synaptic vesicles of normal rat cerebral cortex**, PPT1 was detected as nonglycosylated (31 kDa) and glycosylated (37/39 kDa) forms. On the contrary, only the glycosylated PPT1 was detected on the purified synaptic membranes (*Figure 12*). In comparison, pro-TPP-I (enzymatically inactive precursor, ~67 kDa) seemed to be located both in junctions and vesicles, but the mature TPP-I (43 kDa) resided only in vesicles (unpublished data, *Figure 12*).
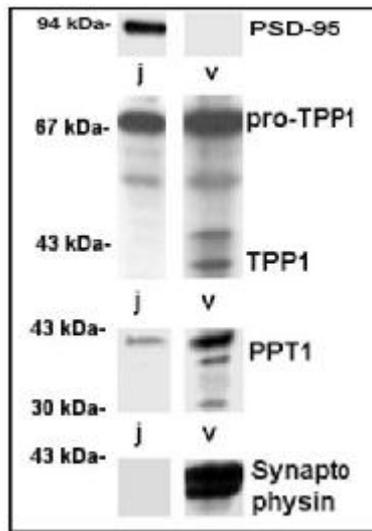
Figure 12. Western blots of synaptic junction (j) and vesicle (v) fractions. Equal aliquots were run on 10% SDS-PAGE and immunopositive bands were detected by ECL. Suopanki et al. 2002 (**IV**) and unpublished.

3. PPT1 expression after excitotoxic insult (IV)

3.1 Expression levels in adult rat brain

Immunohistochemistry and immunofluorescence. Hyperexcitation due to KA affected PPT1 expression in a time-dependent manner. In 24 h, PPT1 immunostaining was enhanced in neurons of amygdala, entorhinal, and pyriform cortex; from 72 h to 1 week, hippocampal pyramidal cells in CA3 and CA1 areas showed distinct enhancement of PPT1 immunoreactivity in the cell soma and neurites. Generally, as indicated by Nissl staining, neurons with increased PPT1 levels resided in those regions, which suffered from seizures. However, the cells showing enhanced expression of PPT1 were not dying cells: PPT1-positive neurons were not collapsed, nor had they pyknotic nuclei or other morphological characteristics of damaged cells. After 1 month, PPT1 returned back to a basal level, except in the CA1 pyramidal neurons. Earlier studies have suggested that CA1 pyramidal neurons remain hyperexcitable and undergo synaptic rearrangements and delayed neuronal damage (Ben-Ari, 1985; Esclapez et al. 1999). In addition, there was a noticeable reappearance of healthy-looking neurons in CA3c area with normal PPT1 levels 1 month after seizure episodes.

Western blotting. Parallel to immunohistochemical analysis, the PPT1 levels were also analyzed using immunoblotting. Proteins extracted from rat hippocampi at 6 h, 24 h, 72 h, 1 week, and 1 month after KA-induced seizures showed a gradual increase in the amount of PPT1 (*Figure 13*). Within 1 week it reached the maximum, being approximately 2.4 times the amount of control PPT1. Thus, keeping in mind the extent of excitoxicity-induced neuron loss in the hippocampus, those neurons remaining must have a significant increase in the PPT1 production. One month after seizures, PPT1 levels were still 1.7 times higher than levels of the equivalent control. The amount of synapsin IIa, which is known to bind small synaptic vesicles, and therefore thought to be an essential protein for synaptic formation (e.g. Sugiyama et al. 2000), peaked within 24 h. The pattern of synapsin IIa upregulation was identical to those reported for early-responding synaptic proteins, which upregulated readily after excitotoxic insult. These were previously analyzed only at the messenger RNA level (reviewed in Zagulska-Szymczak et al 2001).
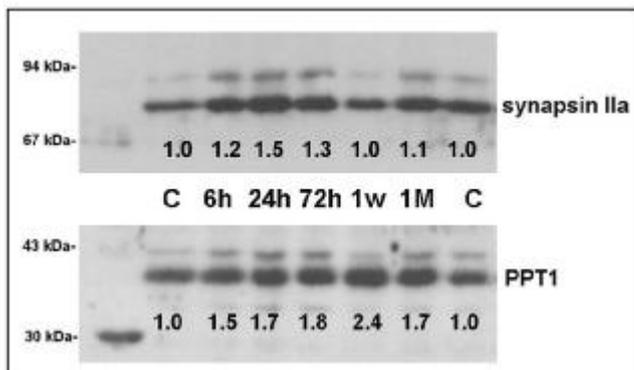
Figure 13. Western blots of hippocampal extracts with anti PPT1 and synapsin IIa. Quantitation included (relative OD values). Suopanki et al. 2002 (**IV**).

3.2 Expression in P15 rat brain

The immature rat brain is more resistant than the adult brain to KA-induced damage up to the end of the third week of life (Tremblay et al. 1984, Ben-Ari 1985). The effect of brain maturation on PPT1 upregulation was studied at P15 rats. The KA-treatment caused epileptic seizures within 1,5 hours, although some individual variation concerning the severity of the seizures, and the seizure frequency was observed. In accordance with earlier reports (Tremblay et al. 1984, Ben-Ari 1985), the brains showed no neuronal damage. As assumed, PPT1 expression was not enhanced anywhere in the brains of rat pups at 24 h or 72 h after injection of KA.

3.3 Influence to PPT1 localization

Immunofluorescence. Since the intensity of PPT1 staining was highest in the CA3 pyramidal cell layer within 1 week after the insult, this time point was chosen for a closer analysis. Compared to control CA3 pyramidal neurons, some changes were detected after epileptic seizures: colocalization of PPT1 with the lysosomal and the

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synaptic marker proteins was more prominent in the epileptic hippocampi than in the control hippocampi. However, PPT1 still colocalized best with NMDAR2B, both in the cell soma and neuritic extensions.

Western blotting. Because the major neuronal response to hyperexcitation and PPT1 upregulation occurred in the hippocampal pyramidal neurons 1 week after KA-induced seizures, hippocampi of that time point were also analyzed by immunoblotting. Rat hippocampi with or without KA-treatment were freshly dissected and fractionated to subcellular components. Control rats, as well as rats with only mild behavioral changes (staring spells and head nodding), showed hardly any PPT1 in the hippocampal synaptic junction. In contrast, rats with full-scale status epilepticus showed, as already visualized in paraformaldehyde-fixed brain slices, that the amount of PPT1 had increased, and was enhanced in the synaptic junctions. Sarcosyl treatment released PPT1 from the synaptic junctions, suggesting a presynaptic localization.

3.4 Hippocampal slice culture – *in vitro* model of excitotoxicity

As an alternative model of excitoxicity, hippocampal slice cultures provided a system for more direct neurotoxin manipulations than rats. Hippocampal slices are known to mature during culturing in a comparable way to *in vivo* conditions (Mueller et al. 1993). Usually, hippocampi from P11-13 rats were sliced and maintained 10 days in culture, and slices were incubated in KA -or glutamate- containing medium in varying conditions. Moreover, hippocampal slices of different ages were tested.

Taking into consideration the amount of neuronal loss in the hippocampal slices already after 2-5 hours of neurotoxin wash-out (Lahtinen et al. 2001), stable PPT1 amounts were regularly detected by Western blotting. In comparison, the same immunoblots first analyzed for PPT1 expression were restained for NMDAR2B, SNAP-25 and TPP-I. Our preliminary results showed that both glutamate and KA inhibited the NMDAR2B expression and also diminished SNAP-25 protein production. The amount of pro-TPP-I was decreased, but the amounts of mature TPP-I form were barely visible and barely unchanged (*Figure 14*, unpublished).
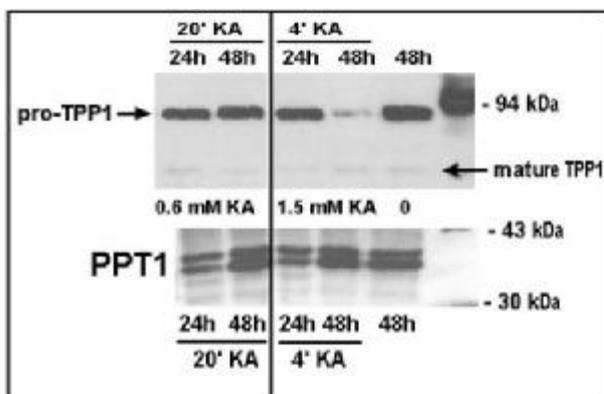

Figure 14. Western blots of hippocampal slices incubated with or without KA. Left panel: 0.6 mM KA, 20 min., right panel: 1.5 mM KA, 4 min. or without KA (0). After washing out KA, the slices were incubated for 24 or 48h. Immunopositive bands of PPT1 and TPP-I were visualized by ECL.

DISCUSSION

1. Methodological aspects

RT-PCR

RT-PCR is an extremely sensitive method for analyzing mRNA levels from tissues and cells. For reliable results, both the elimination of DNA contamination from the RNA template extract and the careful design of primers preferably spanning sequences of adjacent exons were necessary. Proper controls were also included in every set of experiments. Each RT-PCR series was performed six times for samples of neuronal origin and at least three times for noneuronal tissue samples. When drastic changes in PPT1 mRNA levels were detected, four to six RNA extracts representing samples from different individuals were analyzed. All RT-PCR assays performed for this thesis resulted in repeatable and successful analyses. Hence, RT-PCR turned out to be a quick and stable method for the small sample quantities used in this study. *In situ* hybridization, on the other hand, would have provided information about cellular localization and distribution of PPT1 mRNA. *In situ*, being less sensitive than RT-PCR, might have given underestimated results of the mRNA levels in total, thus possibly missing those scarce early embryonic levels detected by RT-PCR.

The specificity of PPT1 antiserum

Three different polyclonal rat PPT1 antisera were tested to identify PPT1 in the studies described. One was an affinity purified PPT1 antiserum, which was a kind gift from Professor Sandra Hofmann (Southwestern Medical Center, University of Texas). Two other ones were raised against a truncated form of PPT1 (amino acids 30-250), which was overexpressed in *E.coli* (I). All three antisera recognized the major PPT1 bands in Western blotting. One of our own antiserum showed the least nonspecific background staining and therefore was chosen for the further Western analyses. This antiserum also recognized the unidentified 70 kDa band, which was detected exclusively from brain extracts of developmental series, and always followed the major PPT1 bands (I, III). This may be due to instant freezing of these samples by liquid nitrogen that was possibly able to preserve proteins or protein interactions in a different way. The gift antiserum, however, showed the same results in immunohistochemistry and immunofluorescence (I, IV), as our own antiserum.

mRNA vs protein

As learned, from, for example, the developmental expression studies of numerous synaptic vesicle proteins (see page 25), mRNA levels do not necessarily correlate with protein levels. Recent proteome studies clearly show that the protein levels cannot be predicted from the mRNA transcript levels (Anderson and Seilhamer 1997, Gygi et al. 1999). This also seems to be true in the case of PPT1 expression in the rat brain. The mRNA expression of PPT1 begins early at E11 and continues with varying levels under strict developmental control. The protein expression is first detected at E16 and it increases steadily without correlating to the prenatal or early postnatal mRNA expression levels of PPT1. Likewise, KA-treatment is known to induce a wide selection of genes at their transcriptional level (see page 33), but changes in mRNA levels do not necessarily lead to changes in protein levels. The degradation rates of pre-existing mRNA pools in the cell soma or synapses can be slowed down without affecting translation rates, or local translation can be sped up without the need for increasing mRNA production. It has been shown that different mRNA pools are present at synapses and isolated subcellular fractions enriched in dendrites are able to synthesize proteins locally based on developmental demands (e.g. Irwin 1985, Villanueva and Steward, 2001). Hence, in the KA-study (IV), detecting proteins reflects better the physiological events in the hyperactive neurons than merely measuring the levels of mRNA.

Isolation methods

Isolations of subcellular compartments of the synaptic region (synaptic vesicles, synaptic junction, PSD) were done according to the well-known extraction method (e.g. Carlin et al 1980, Cho et al. 1992), where stepwise centrifugation and changes in buffer conditions removed extracellular components and unwanted subcellular organelles, such as lysosomes and mitochondria. The purity of samples was also checked by EM (*Figure 11*, page 46). In addition, the presence of significant amounts of lysosomal or mitochondrial proteins in the final fractions was excluded by reanalyzing the immunoblots with specific monoclonal antibodies against the marker proteins.

By immunofluorescence PPT1 was found to colocalize with NMDAR2B, the subunit of NMDA-receptor, indicating localization in the synaptic junction. The resolution of immunofluorescence, however, cannot distinguish pre- and postsynaptic sides of the junction. Therefore, to determine the localization of PPT1 in the synapse, it was important to use alternative methods. The detergent treatments of synaptosomes were performed for obtaining the pure synaptic junctions (*Figure 11,* page 46). Further sarcosyl treatment of synaptic junctions releases tightly attached proteins, including components, which hold pre- and postsynaptic membranes together, and results in further purified PSDs composed of crosslinked scaffold-proteins. These treatments may

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remove some important synaptic components (e.g. possible signaling complexes attached to the membrane), and no clear-cut separation between pre- and postsynaptic molecules can be obtained. But, in combination with imaging techniques the analysis of subcellular compartments will give valuable information about molecular events in neurons.

2. Developmental aspects

INCL-pregnancy does not differ from healthy pregnancy, although abnormal storage material is present in the fetus (Rapola et al. 1990 & 1993). Neurological development continues normally until 5 months of age and the earliest clinical signs of INCL appear at a mean age of 9.5 months. In the developing human brain, synaptic connections form exuberantly during the third trimester of gestation and early childhood (see page 18). Clearly, INCL-onset coincides with the ongoing synaptogenesis. Synapse formation in the rat cerebral cortex starts at birth and peaks around P26. At P12-P30, neuronal connectivity increases 10-fold (Eayrs and Goodhead, 1959; page 19). The developmental data from rat (I-III) and mouse (Isosomppi et al. 1999) PPT1 expression show that the major increase in PPT1 production occurs during synaptogenesis. Overproduction of synapses at that time could be in agreement with increased PPT1 expression, particularly since PPT1 has been localized in the CNS synapses of the adult rodent brain (IV, Lehtovirta et al. 2001). Furthermore, the eye-opening period in rodents boosts synaptic activities, which could lead to the increase in PPT1 mRNA levels detected during that period (II). In this study, the subcellular distribution of PPT1 during development was not studied, but the preliminary results of Kopra et al. have shown gradual appearance of PPT1 in developing retinal axons and in axonal varicosites (Kopra O et al. poster 670.17 at Neuroscience 2001 Conference, San Diego). Like dendritic shafts, axonal varicosites may or may not develop into synapses depending on the stability of the formed neuronal connections (see page 27). During early synaptogenesis, synaptic connections are in a constant reshaping mode, degenerating and regenerating with falling or rising synapse densities. Numerous, overactive early synapses might be able to compensate for the putative functional impairment of synapses caused by the PPT1 deficiency. Thus, later synaptic pruning could uncover existing impairments and lead to overpruning and loss of synaptic and neuronal connections.

Neuron-specific role of PPT1 is further supported by the fact that developmental expression pattern of PPT1 in nonneuronal tissues did not change (II). PPT1 expression showed a stable pattern in both mRNA and protein levels. Nonneuronal tissues have also

been shown to have storage material without any cellular destruction or tissue malfunction (e.g. Rapola 1993).

3. Action of PPT1 in synapses?

Lysosomal storage material in INCL-lymphoblasts can be dissolved by extracellular PPT1, which is internalized mainly via Man 6-P-pathway (Lu et al. 1996). In different cell types, however, lysosomal trafficking can vary and be completely independent on Man 6-P-pathway (e.g. Rijnboutt et al. 1991, Tanaka et al. 2000). In I-cell disease fibroblasts, the newly synthesized lysosomal enzymes are secreted and only a 5-20% fraction is targeted to lysosomes due to the phosphotransferase deficiency (Hasilik and Neufeld, 1980). In the I-cell disease brain, as well as in certain other organs, however, lysosomal enzyme levels are normal (Kornfeld and Sly, 1995). Knockout mice lacking the two Man 6-P-receptor types of mammalian cells have also shown normal levels and activities of several lysosomal enzymes in the brain, but reduced levels in the fibroblasts (Dittmer et al. 1999). Thus, the mechanism of PPT1 delivery and action might well be different in neurons from that in other cell types.

Normally, PPT1 is transported to nerve terminals and, as the present study shows, there it is localized in synaptic vesicles and synaptic membranes (IV, Heinonen et al. 2000, Lehtovirta et al. 2001). Recently, the disease phenotype and intracellular localization of PPT1 were found to correlate only in neuronal cultures. In contrast to the severely malfunctioning PPT1 staying in the ER at the cell soma and causing INCL, the deficient enzymes responsible for late-onset CLN1 were found in the neurites and terminals. Furthermore, despite the localization of the mutated enzyme, its *in vitro* depalmitoylation activity was low (Salonen et al. 2001). Thus, possibly the threshold level of PPT1 is small needed for maintaining early synaptic functions that would explain the late-onset phenotype. Finding PPT1 in synaptic vesicles and synaptic junctions supports the neuron-specific function of PPT1 and selective manifestation of the disease in the CNS. Intriguingly, *in vitro* palmitoylation activity was recently found in synaptic vesicles and this activity was developmentally regulated. Among approximately 10 palmitoylated proteins were the SNARE-proteins synaptotagmin and synaptobrevin, which were palmitoylated only in vesicles purified from the adult rat brain, thus suggesting a developmental switch in palmitoylation (Veit et al. 2000). If palmitoylation occurred locally in synapses for developmental needs or quick local needs in recycling vesicles or receptors, it would be logical to find depalmitoylation activity of PPT1 there too.

Several cellular functions, such as signal transduction, vesicular transport and maintenance of cellular architecture, depend on post-translationally lipid-modified proteins (e.g. Schmidt 1989). Neuron-specific proteins, such as PSD-95 and growth-associated-protein 43, (GAP-43) are palmitoylated (Goslin et al. 1988, Topinka and Bradt 1998). Palmitoylation of SNARE-proteins synaptobrevin and SNAP-25, as well as palmitoylated synaptotagmin might contribute to the regulation of the synaptic vesicle cycle (Hess et al. 1992, Chapman et al. 1996, Veit et al. 1996 & 2000). Ha-Ras, an intracellular palmitoylated and farnesylated protein, is one of the *in vitro* substrates of PPT1 (Camp and Hofmann 1993). Ha-Ras with palmitate alone is able to modulate multiple effector proteins that induce neuronal differentiation (e.g. Booden et al. 1999 & 2000), thus emphasizing the significant biological role of palmitoylation in Ha-Ras. A direct *in vivo* interaction between PPT1 and these putative substrate candidates has yet to be studied. Due to the reversibility of palmitoylation and quick *in vivo* turnover of palmitate (e.g. 20 min in Ha-Ras; Magee et al. 1987) the enzyme-substrate interaction can only be transient, and is also modified by other factors.

Synaptic hyperactivation induced PPT1 expression in hippocampal pyramidal cells of the rat brain (IV). The protein levels stayed elevated in CA1 hippocampal neurons, which are known to remain hyperexcitated (Ben-Ari 1985, Esclapez et al. 1999), even one month after seizure episodes. PPT1 could be responsible for protecting neurons from overexcitation, either balancing or diminishing excitation. Alternatively, PPT1, which may be needed for creating/maintaining new synaptic connections that are known to rise after hyperexcitation, could participate in neosynaptogenesis. Whether the enhanced PPT1 synthesis was local remains to be investigated. Interestingly, PPT1 was found to colocalize with the glutamatergic receptor subunit, NMDAR2B, in normal and hyperactivated neurons, thus suggesting that PPT1 might act via glutamatergic pathway. This pathway involves a series of second messengers, modulators, and activators, which come into contact with NMDA-receptors in postsynapse for propagating the excitation signal further downstream. Some studies have shown that NMDA-receptors can also localize in presynaptic membranes of certain neuron types (page 20). Activation of presynaptic NMDA-receptors was shown to cause neuronal depression by inhibiting neurotransmitter release (Sequeira et al. 2001).

Interestingly, hyperexcitation enhanced the association of PPT1 with the presynaptic membrane. This might simply be a consequence of enhanced exocytosis of synaptic vesicles during hyperexcitation. Alternatively, association of PPT1 with synaptic membranes might be an essential step in the action of PPT1 in synapses. These findings should be confirmed under more physiological conditions.

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Recently, scientists at the Southwestern Medical Center (University of Texas) managed to produce PPT1-knockout mice. The mice reproduced the human phenotype (CLN1); autofluorescent storage material had GROD-type ultrastructure in the brain, and neuronal loss in the cerebrum and cerebellum was prominent. Yet, the PPT1-knockout mice died relatively late (7-10 months of age) compared to humans with CLN1 (Gupta et al. 2001). Thus, the mouse brain was still able to develop relatively normally without PPT1, either because of enzymes compensating for the absence of PPT1, or the mouse brain development does not need PPT1 as early as the developing human brain does. After all, already at birth, the human brain is more developed than the mouse brain. As reported, PPT1 mutations cause a variety of human phenotypes with infantile to adult onset. Thus, rather than being a model of INCL, this could be a model of an adult onset CLN1-phenotype.

4. Therapy for INCL?

Some symptomatic therapies for INCL-patients are available, but they are not curative. Recently tested hematopoietic stem cell transplantation in three INCL patients in Finland was able to normalize PPT1 activity in peripheral leukocytes, but the activity remained low in cerebro-spinal fluid. Transplantation postponed the progress of the disease, but eventually all three patients continued to develop INCL one or two years after receiving transplants (Lönnquist et al. 2001). Another therapeutic attempt showed that a largely used and well-characterized lysosomotrophic drug, phosphocysteamine, was able to deplete lysosomal ceroids in CLN1-lymphoblasts. Moreover, it was able to prevent re-accumulation and inhibited apoptosis. The effect of phophocysteamine was phenotype-specific, that is not all CLN1 cell lines responded equally. Besides, the continuous presence of phosphocysteamine was needed in order to prevent re-accumulation (Zhang et al. 2001). Lymphoblasts are not equal to neurons and, as Zhang et al. pointed out, CLN1 pathogenesis might not be the simple result of deficiency in the catalytic activity of PPT1. Presently, the phophocysteamine is being tested in experimental animals (e.g. PPT1-knockout mice).

Laboratories around the world are trying to develop more effective means to deliver curative molecules to the CNS neurons. The choices range from neural grafts and inducible virus-assisted gene transfer to carrier-mediated drug absorption (Janson et al. 2001, Temisamani et al. 2001, Yang et al. 2001). Depending on the type of the neurodegenerative disease, numerous factors, not least of which is the time of onset, have to be considered before deciding whether to prevent, stop or reduce the cell loss

rate. Alternative methods, including usage of neuroprotective agents or neurotrophic factors, could be preventive in certain diseases.

Would gene therapy be successful in INCL? Viral vectors carrying the PPT1 gene would be able to pass the blood-brain-barrier and selectively infect certain neurons early enough. Such therapy would probably require continuous boost injections, since the turnover of PPT1 observed in overexpression systems is only 3-5h (Das et al. 2001). 'Slow release polymers', matrices introducing corrective proteins for longer periods of time, or secretory cell lines overproducing PPT1 could provide other options for therapy design. Again, the half-life of the protein is critical as is its stability in neurons, and in different neuron subtype-specific functions. Once the pathogenesis is better understood, the development of new therapies will become possible.

Gene therapy made a major step forward when young patients with Canavan disease received virus particles infused directly into certain brain areas (The Scientist 15:20, 2001; Leone et al. 2000). Recombinant viruses were carrying an enzyme, aspartoacylase (ASPA), the deficiency of which leads to neuron and oligodendrocyte damage, demyelination and brain atrophy in the CNS of Canavan children. Most Canavan patients die by 10 years of age. The locally supplied treatment was reported to diminish some symptoms, but long-term functions are yet to be seen. Several questions remain. How long is the targeted and localized virus infusion able to diminish the progress of the disease? How long does the transgene stay functional in humans? Are the virus transgenes integrating into a neuron's genome or do they remain episomal.

The future is ours to see.

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ACKNOWLEDGEMENTS

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