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Systematic Analysis of disease pathways in Congenital, Infantile and Juvenile Neuronal Ceroid Lipofuscinoses

Enzo Scifo

ACADEMIC DISSERTATION

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To my mother

“As long as our brain is a mystery, the universe, the reflection of the structure of the brain will also be a mystery.”

--*Santiago Ramon y Cajal*

Abstract

Neuronal ceroid lipofuscinoses (NCL) are the most common inherited progressive encephalopathies of childhood. NCL disease genes were first identified in 1995 and since then, nearly 400 mutations (mostly autosomal recessively inherited) in thirteen known genes (*CLN1-8*, *CLN10-14*; http://www.ucl.ac.uk/ncl) have been described. Despite progress in the NCL field, the primary function and physiological roles of most NCL proteins remain unresolved. In this thesis we employed systematic approaches, including: functional proteomics, bioinformatics and mouse disease models, in an effort to clarify disease pathways associated with congenital, infantile and juvenile NCL in the human brain. We focused on four disease genes with different ages of onset: *CLN10* (congenital), *CLN1* (infantile classic, late infantile, juvenile, adult), *CLN3* (juvenile, classic) and *CLN5* (late infantile variant, juvenile, adult).

First, we systematically examined the synaptic proteome in a cathepsin D *(Ctsd / Cln10)* knockout ($Ctsd^{-/-}$) mouse model of congenital NCL (CLN10 disease), where the synaptic pathology resembles that of patients. Mouse brain synaptosomal fractions isolated from $Ctsd^{-/-}$ knockout and control mice, were analysed by quantitative mass spectrometry. This work yielded nearly 600 identified proteins from three biological replicate measurements, out of which 43 proteins were differentially expressed in the $Ctsd^{-/-}$ mice. We utilized protein-protein interaction (PPI) databases to connect and bridge the differentially expressed proteins then overlaid the resulting network with brain specific expression data from the Human Gene Atlas. The network of differentially expressed proteins was subjected to Gene ontology, pathway analysis and checked for involvement in disease phenotype. Finally, a subset of the data was systematically validated by quantitative Western blotting, immunohistochemistry and immunofluorescence confocal microscopy. This work highlighted defects in migratory functions of cathepsin D deficient cells that were attributed to downregulation of cytoskeletal proteins.

Secondly, we aimed to map the CLN3-CLN5 protein interactome in the brain by identifying their associated proteins. We isolated and identified protein complexes from SH-SY5Y stable cells, followed by stringent filtration with *S*ignificance *A*nalysis of *INT*eractome (SAINT), functional assignment by bioinformatics and validation analyses to unravel molecular mechanisms underlying CLN3 and CLN5 diseases. This work revealed 42 / 31 novel CLN3 / CLN5 interacting partners (IP), respectively. The extent of crosstalk (several bridging IP) amongst CLN3 and CLN5,

suggests that the mechanisms leading to the functional deficits are shared between them. CLN3 was implicated in new roles of G-protein signalling and protein folding / sorting in the ER.

Finally, we isolated protein complexes from human PPT1 (CLN1) expressing SH-SY5Y stable cells by single step affinity purification and subjected them to filter assisted sample preparation, prior to analysis by mass spectrometry. The goal of this study was to identify *in vivo* PPT1 substrates that could provide insight on the onset and progression of CLN1 disease. Findings from our proteomic analysis of the human PPT1 interactome support suggested roles of the protein in axon guidance and lipid metabolism, as well as point to putative new roles of PPT1 in neuronal migration and dopamine receptor mediated signalling pathway.

Résumé

Les céroïde-lipofuscinoses neuronales (CLN) sont les encéphalopathies progressives héréditaires les plus fréquentes chez l'enfant. Les gènes responsables de la maladie CLN ont été identifiés en 1995 et depuis, près de 400 mutations (principalement autosomiques récessives) dans treize gènes connus (*CLN1-8, CLN10-14*; http://www.ucl.ac.uk/ncl) ont été décrites. Malgré les progrès réalisés dans le domaine des CLN, la fonction principale et les rôles physiologiques de la plupart des protéines impliquées dans les CLN restent indéterminés. Les travaux présentés dans cette thèse ont contribué à clarifier les mécanismes associés aux CLN congénitales, infantiles et juvéniles dans le cerveau humain en utilisant des approches systématiques telles que, la protéomique fonctionnelle, la bio-informatique, ainsi que par l'étude de modèles de la maladie chez la souris. Nous nous sommes concentrés sur quatre gènes de la maladie auxquels correspondent différents âges de développement: *CLN10* (congénitale), *CLN1* (classique infantile, infantile tardive, juvénile, adulte), *CLN3* (juvénile, classique) et *CLN5* (variante infantile tardive, juvénile, adulte).

Afin de mieux comprendre les disfonctions liées à la maladie CLN10, nous avons réalisé une étude du protéome synaptique de souris cathepsin *Ctsd / Cln10* knockout (*Ctsd−/−*), c'est à dire de souris modèles NCL congénital pour lesquelles les pathologies sont similaires à celles de patients humains atteints de la maladie CLN10. Dans un premier temps, l'analyse par spectrometrie de masse de fractions synaptosomales isolées de de souris controles et de souris *Ctsd−/−* knockout a permis d'identifier 600 protéines parmis lesquelles 43 sont exprimées de facon differentielle chez les souris *Ctsd−/−* knock-out. Des connections entre ces différents candidats ont ensuite été établies en utilisant la base de données PPI (protein-protein interaction) et ces résultats ont été corrélés avec les données provenant du Human Gene Atlas concernant les protéines exprimées spécifiquement dans le cerveau. Le réseau ainsi créée a été annotaté avec les termes de Gene ontology, selon leur appartenance èa des voix de signalisation connues et aussi en fonction de leur implication dans des phenotypes liés aux maladies. Afin de valider ces résultats bio-informatiques, certains candidats ont été testés par immunobavargae quantifitatif, immuno-histo-chime et microscopie confocale. En conclusion, ce travail aura permis de mettre en évidence les défauts de migrations dans les cellules déficientes en Cathepsin D due à une dérégulation des protéines des cytosquelettes.

Deuxièmement, nous avons cherché à cartographier l'interactome des protéines qui interagissent avec CLN3 et CLN5. Nous avons utilisé la technique TAP (Tandem

Affinity Purification) dans les cellules de neuroblastome humains. Les protéines identifiées par SM ont ensuite été analysées à l'aide du logiciel SAINT (*S*ignificance *A*nalysis of *INT*eractome). L'affectation fonctionnelle des protéines identifiées par la bio-informatique et la validation des analyses a permis de démêler les mécanismes moléculaires sous-jacents des maladies de CLN3 et CLN5. Ce travail a révélé 42 et 31 nouvelles CLN3 / CLN5 partenaires d'interaction, respectivement. Le faite que de nombreuses protéines aient été identifiées lors des essais avec CLN3 et CLN5, suggère que les mécanismes conduisant à des déficits fonctionnels sont communs aux deux. L'étude a mis en évidence l'implication de CLN3 dans petite protéine G de signalisation ainsi que le repliement des protéines au niveau du RE dans cette maladie.

Enfin, nous avons isolé des complexes protéiques de PPT1 (CLN1) chez l'homme à partir de cellules SH-SY5Y-PPT1-CTAP-Puro stables utilisant la purification par affinité. Ces derniers ont ensuite été préparés selon la technique FASP (*F*ilter *A*ssisted *S*ample *P*reparation*)* avant d'être analysé à l'aide de la spectrométrie de masse. L'objectif de cette étude était d'identifier les substrats (*in vivo*) de PPT1 qui peuvent nous éclairer sur l'apparition et la progression de la maladie de CLN1. Les conclusions de l'analyse protéomique de l'interactome PPT1 suggèrent que ce complexe serait impliqué dans la migration neuronale et les voies de signalisation du récepteur de la dopamine.

Contents

List of original publications

This thesis is based on the following publications:

I Koch, S.*, **Scifo, E.*,** Rokka, A., Trippner, P., Lindfors, M., Korhonen, R., Corthals, G., Virtanen, I., Lalowski, M., Tyynelä, J. Cathepsin D deficiency induces cytoskeletal changes and affects cell migration pathways in the brain. *Neurobiol Dis.* 2013 Feb; 50:107-19. doi: 10.1016/j.nbd.2012.10.004. Epub 2012 Oct 12. (*Equal contribution)

II **Scifo, E.,** Szwajda, A., Debski, J., Uusi-Rauva, K., Kesti, T., Dadlez, M., Gingras, A-C., Tyynela, J., Baumann, M. H., Jalanko, A., Lalowski, M. Drafting the CLN3 Protein Interactome in SHSY5Y Human Neuroblastoma Cells: A Label-free Quantitative Proteomics Approach. *J Proteome Res.* 2013 May 3; 12(5):2101-15. doi: 10.1021/pr301125k. Epub 2013 Apr 19.

III **Scifo, E.,** Szwajda, A., Soliymani, R., Debski, J., Uusi-Rauva, K., Dadlez, M., Gingras, A-C., Tyynelä J., Jalanko, A., Baumann, M. H., Lalowski, M. Proteomic Analysis of the Palmitoyl Protein Thioesterase 1 Interactome in SHSY5Y Human Neuroblastoma Cells. *Manuscript (J Proteome Res 2014)*

The publications are referred to in the text by their roman numerals.

Abbreviations

aa, Amino acid

AAA, ATPases associated with a variety of activities

AAV(s)/ 2/ 5/ rh.10, Adeno-associated viral vectors/ serotypes: 2/ 5/ rhesus 10

ACN, Acetronitrile

AMPA, α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AP-MS, Affinity purification coupled to mass spectrometry

ARD1, ADP-ribosylation factor domain protein 1

ATP13A2, ATPase type 13A2

BBB, Blood brain barrier

BSA, Bovine serum albumin

cAMP, Cyclic adenosine monophosphate

CAPs, Cancer-associated proteins

CDC48, Cell division protein 48

CLN1-14, Ceroid-lipofuscinosis neuronal proteins 1-14

CNPase, 2',3'-Cyclic-nucleotide 3'-phosphodiesterase

CNS, Central nervous system

CoA, Co-enzyme A

CSPα, Cysteine-string protein-α

CTAP-Puro, Expression plasmid pES-CTAP-Puro

CTSF, Cathepsin F

$D_{1\text{-}5}$, Dopamine receptor subtypes (1-5)

DA, Dopamine / dopaminergic

DAT, Dopamine transporter

DAVID, Database for annotation, visualization and integrated discovery

DLAT, Dihydrolipoamide S-acetyltransferase

DLD, Dihydrolipoamide dehydrogenase

DOMON, Dopamine β-mono-oxygenase N-terminal domain

EGIS-8332, [+/−]-7-acetyl-5-[4-aminophenyl]-7,8-dihydro-8-cyano-8-methyl-9H-1,3-dioxolo-[4,5-h]-2,3-benzodiazepine

EPMR, Epilepsy with progressive mental retardation

GD1, Type 1 Gaucher's disease

GRN, Granulin

ERT, Enzyme replacement therapy

FAD^+, Flavin adenine dinucleotide (oxidized)

FAK, Focal adhesion kinase

FASP, Filter assisted sample preparation

FDR, False discovery rate

GFP, Green fluorescent protein

Gi/o, Inhibitory G proteins

GO, Gene ontology

GRODS, Granular osmiophilic deposits

Gs/olf, Stimulatory G proteins

hCLN3, Human CLN3

HD, Huntington's disease

HEK 293, Human embryonic kidney 293

ICAT, Isotope coded affinity tags

IF, Immunofluorescence

IMPase, Inositol monophosphatase

IP, Interacting partners

ITGB, β1-integrin

iTRAQ, Isobaric tags for relative and absolute quantification

JME, Juvenile myoclonic epilepsy

JNCL, Juvenile NCL

KCTD7, Potassium channel tetramerisation domain containing 7

LAMP1, Lysosomal-associated membrane protein 1

LSD, Lysosomal storage disorder

MAP1B/ 2, Microtubule associated protein 1B / 2

MBP, Myelin basic protein

'mnd', Motor neuron disease

MS3, Triple stage mass spectrometry

NAD^+, Nicotinamide-adenine dinucleotide (oxidized)

nano-LC /ESI/MS/MS, Nano-flow liquid chromatography / electrospray ionization / tandem mass spectrometry

NAT1, N-terminal acetyltransferase 1

NCL, Neuronal ceroid lipofuscinoses

NTAP, Expression plasmid pCeMM-NTAP (GS)Gw

p97, 97 kDa protein

PA, Protein A

PDHA1, Pyruvate dehydrogenase (lipoamide) alpha 1

PDHB, Pyruvate dehydrogenase (lipoamide) beta

$PDH_{c,}$ Pyruvate dehydrogenase complex

PIP(2), Phosphatidylinositol 4,5-bisphosphate

PLCβ, Phospholipase Cβ

PLP1, Proteolipid protein 1

PNMT, Phenylethanolamine N-methyltransferase

POLG1, Mitochondrial DNA polymerase gene

PPI, Protein-protein interactions (PPI)

PPT1, Palmitoyl protein thioesterase 1

OMIM, Online mendelian inheritance in man

RNAi, RNA interference

RRF, Retrorubal field

SBP, Streptavidin binding peptide

SCMAS, Subunit c of mitochondrial ATP synthase

SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SILAC, Stable isotope labelling by amino acids in cell culture

SNc, Substantia nigra pars compacta

TAP-MS, Tandem affinity purification coupled to mass spectrometry

TER94, Transitional endoplasmic reticulum 94

TEV, Tobacco etch virus

TH, Tyrosine hydroxylase

TLN, Talin

TMT, Tandem mass tags

TPP1, Tripeptidyl peptidase 1

VCP, Valosin containing protein

VEM, Virtual expert mass spectromist

VM, Ventral midbrain

VPM/ VPL, Ventral posterior medial / lateral nucleus of thalamus

VTA, Ventral tegmental area

$vLINCL_{Fin}$, Finnish variant late infantile neuronal ceroid lipofuscinosis

XLMR, X-linked mental retardation

Y2H, Yeast two hybrid

Introduction

The brain is the command centre of the nervous system in all vertebrates and most invertebrates (with a few exceptions e.g. jelly fish, sponges). Structurally, the human brain is similar to other mammalian brains, except that it is more "encephalised" i.e. is relatively larger in proportion to body size (Hofman, 2014). Given its complexity, understanding how the human brain functions requires studies at different spatial and temporal scales: from genes, proteins, synapses and cells to microcircuits, brain regions and the whole brain, at varying stages. Invasive techniques on humans are limited because of ethical issues, hence the use of model organisms (e.g. mouse, rat, zebra fish) which can easily be manipulated to provide insight into the function of the brain.

Neuronal ceroid lipofuscinoses (NCL) which are the focus of this thesis, are mostly childhood-onset autosomal recessively inherited neurodegenerative disorders (Mole SE, 2011). They are characterised by early accumulation of auto-fluorescent storage material in lysosomes of neurons or other cells and degeneration of cortical neurons. NCL patients suffer from progressive loss of vision, mental and motor deterioration, epileptic seizures and premature death (Haltia, 2006). NCL are caused by mutations in 13 known genes (*CLN1-8, CLN10-14*) (http://www.ucl.ac.uk/ncl), some of which have only been recently identified. The estimated incidence of NCL is 1 per 12,500-200,000 persons in the USA and Northern Europe (Haltia, 2006; Uvebrant and Hagberg, 1997). Although NCL are heterogeneous, they share a similar phenotypic profile which is suggestive for shared molecular mechanisms. These diseases have proven challenging to study with traditional cell biology or biochemistry based methods and are therefore good candidates for systematic approaches that rely on interdisciplinary methods to provide a holistic view of disease pathogenesis. After nearly two decades of NCL research, the precise function or physiological role of most NCL proteins remain unresolved. This thesis work attempts to narrow the knowledge gap through studies of protein-protein interactions (PPI) associated with CLN10, CLN3 / CLN5 and CLN1.

We investigated the mouse brain synaptic proteome of *Ctsd*$^{-/-}$ / *Cln10*$^{-/-}$ knockout using a combination of quantitative mass spectrometry, bioinformatics, quantitative Western blotting and qualitative immunohistochemistry. Protein interaction data was utilised to connect and bridge differentially expressed proteins identified from the cathepsin D knockout mice. The resulting protein map was further enriched with brain specific gene expression, functional ontologies / pathways and disease

phenotype information. This enabled us to construct a dynamic, disease-protein centred network with predicted functional modules. We also mapped the CLN3-CLN5 interactome in the brain by isolating and identifying CLN3 / CLN5 protein complexes from SH-SY5Y stables cells using tandem affinity purification coupled to mass spectrometry (TAP-MS), in concert with stringent *S*ignificance *A*nalysis of *INT*eractome (SAINT), co-localisation and co-immunoprecipitation assays. The study implicated CLN3 in G-protein signalling and protein folding / sorting in the ER. Finally, we isolated protein complexes from human CLN1 (PPT1) expressing stable cells and identified them by single step affinity purification coupled to mass spectrometry (AP-MS). PPT1 IP from this work linked the protein to putative new roles in neuronal migration and the dopamine receptor mediated signalling pathway.

2 Review of the literature

2.1 Functional proteomics and quantitative mass spectrometry

Functional proteomics is the study of protein dynamics, including protein-protein interactions (PPI) and posttranslational modifications (PTM) of individual proteins, in the context of their impact on biological function. Quantitative mass spectrometry (MS) is an unbiased tool for probing protein complexes at near to endogenous levels and determining protein abundance, PPI, or PTM of individual constituent proteins (Aebersold and Mann, 2003; Matthiesen et al., 2011). Label-free quantitative MS was recently utilised to draft a map of the human proteome and create two publicly available database resources (Proteomics DB and human proteome map), for analysis of proteomic datasets from human samples (Kim et al., 2014; Wilhelm et al., 2014). Similarly to the impact of next generation sequencing on genomics and transcriptomics (Stapley et al., 2010), mass spectrometry has revolutionalised proteomic studies. However, MS technology is still limited by its reliance on protein databases with predefined sequences, for identification of proteins. Sample preparation for MS involves several critical features, e.g. sample pre-fractionation, protein digestion, sample clean-up / separation, which precede the MS runs and data analysis.

Several stable isotope labelling methods are currently used for robust measurements of relative protein abundance within complex samples. Examples include: isotope-coded affinity tags (ICAT) (Gygi et al., 1999), stable isotope labeling by amino acids in cell culture (SILAC) (Ong et al., 2002), isobaric tags for relative and absolute quantification (iTRAQ) (Ross et al., 2004), tandem mass tags (TMTs) (Dayon et al., 2008; Thompson et al., 2003) and triple-stage mass spectrometry (MS3) (Ting et al., 2011). A comparison of protein abundance in multiple tissue samples is possible with iTRAQ and TMT labelling, since there is no requirement to grow cells in isotope-containing culture medium (Altelaar et al., 2013). Quantitative MS data from iTRAQ and TMT experiments may be compromised in accuracy or precision due to inefficient labelling and contamination from nearby isobaric ions, which are isolated and fragmented alongside the target ions (Karp et al., 2010; Shirran and Botting, 2010). The use of MS3 and improvements in data analysis should circumvent this problem (Karp et al., 2010; Ting et al., 2011).

Labelling based quantitative MS strategies are limited by: the high cost of reagents, incomplete labelling, increased time / complexity of sample preparation, sample concentration and specific software for analysis (Elliott et al., 2009). As such, label-free quantitative proteomics based on relative quantitation of peptide ion intensity or spectral counts may also be utilised to estimate relative abundance of proteins within a complex sample (Old et al., 2005). Bioinformatic platforms, including: SAINT (Skarra et al., 2011), OpenMS (Kohlbacher et al., 2007; Rost et al., 2014) and MaxQuant (Cox and Mann, 2008; Cox et al., 2009), have been designed for analysis of label-free or isotope-labeled MS data. Virtual expert mass spectrometrist (VEMS) which is a specific software for iTRAQ or TMT data analysis is utilised to obtain more accurate peptide ratios (Matthiesen, 2007; Rodriguez-Suarez et al., 2010).

2.2 Protein-Protein Interaction Networks

Understanding how protein-protein interaction networks can become dysfunctional allows us to apply therapeutic intervention with drugs, genetic or environmental tools. Affinity purification-based methods combined with mass spectrometry and systematic yeast two-hybrid (Y2H) screens (Ewing et al., 2007; Gavin et al., 2006; Gavin et al., 2002; Krogan et al., 2006) have been used to generate large-scale protein-protein interaction (PPI) networks for human (Rual et al., 2005; Stelzl et al., 2005), and several model organisms including *C. elegans* (Li et al., 2004), *Drosophila* (Formstecher et al., 2005; Giot et al., 2003) and yeast (Ito et al., 2001; Uetz et al., 2000). Moreover, medium-scale PPI maps were created for signalling pathways (Colland et al., 2004).

However, the currently available interaction data are static and incomplete, thus inadequate for understanding the function of proteins within their cellular milieu. It is also well understood that methods for the generation of protein interaction data differ to such an extent that they result in complementary rather than overlapping data (Kaltenbach et al., 2007; Zhao et al., 2005). Therefore, to further probe disease pathways at a molecular level, highly focused PPI screens and several confirmation studies that minimize false positives should be performed to refine interaction networks. Integration of interaction data with qualitative and quantitative information (gene expression / localisation studies and bioinformatics), is necessary to construct maps of protein function that reflect dynamic cellular processes (Figeys, 2008; Stelzl and Wanker, 2006).

2.2.1 Protein connectivity in oligogenic and complex diseases

Although the onset and progression of several monogenic diseases (e.g. phenylketonuria and cystic fibrosis) was once attributed to a single gene (Mendelian Inheritance), they have since proven to be oligogenic, i.e. modulated by one or more modifier genes (Badano and Katsanis, 2002). Causative genes associated with both oligogenic and complex diseases usually have similar cellular functions and overlapping biological modules or pathways (Badano and Katsanis, 2002; Oti and Brunner, 2007), thus providing a strong correlation between protein connectivity and disease. Structural studies on a subset of disease genes, suggest that their phenotypes are associated with mutations that probably perturb the quaternary structure of the proteins and consequently disrupt protein-protein interactions (Ferrer-Costa et al., 2002). A survey of mutations that disrupt protein interactions in 65 diseases, showed most of them to have a loss of function phenotype often attributed to disturbed transient protein-protein interactions (Schuster-Bockler and Bateman, 2008).

Human protein-protein interaction datasets from large-scale experiments (Rual et al., 2005; Stelzl et al., 2005) and literature mining (Chatr-aryamontri et al., 2007; Kerrien et al., 2007), have provided a global view of disease gene networks. Inherited disease genes have often been assumed to encode proteins with a larger number of interactions in comparison to non-disease genes and thereby favor interactions with other disease gene products (Feldman et al., 2008; Gandhi et al., 2006; Goh et al., 2007), hence forming the hubs within interaction networks (Xu and Li, 2006). However, recent evidence suggests that the "hub-ness" of inherited disease genes maybe apparent. Exclusion of essential genes from analysis of interaction networks, disassociates hubs from disease genes and shifts non-essential disease genes to the periphery of the network, where they have a neutral effect on a cell's survival (Goh et al., 2007).

2.2.2 Interactome networks in human health and disease

High-throughput interactome studies of a few human diseases where interaction data is of sufficient quality are useful in understanding underlying disease mechanisms (Rual et al., 2005; Stelzl and Wanker, 2006). The first large-scale affinity purification mass spectrometry of PPI in human cells (Ewing et al., 2007) identified 6463 high-confidence interactions of particular relevance to various human diseases.

A method for purification of native protein complexes at sub-endogenous levels (tandem affinity purification, TAP) was first described in yeast and later successfully applied to mammalian cells (Burckstummer et al., 2006; Puig et al., 2001). In TAP, a target protein fused to a protein tag is expressed in a cellular or organism host, for purposes of isolating it along with associated interacting partners. Extracts from the host cell or organism are utilised to isolate protein complexes, at near to endogenous levels in dual affinity purification steps (Burckstummer et al., 2006; Rigaut et al., 1999). Similarly to co-immunoprecipitation (affinity matrix-antibody), TAP is dependent on specific high affinity protein-protein (affinity matrix-tag) interactions for isolation of *in vivo*-associated target protein complexes from extracts (Puig et al., 2001; Rigaut et al., 1999). Unlike co-immunoprecipitation, the various steps in TAP (Figure 1) ensure reduced background contamination from abundant cellular proteins. As such, highly purified protein complexes present at low concentrations can be isolated from the starting material (Puig et al., 2001; Rigaut et al., 1999).

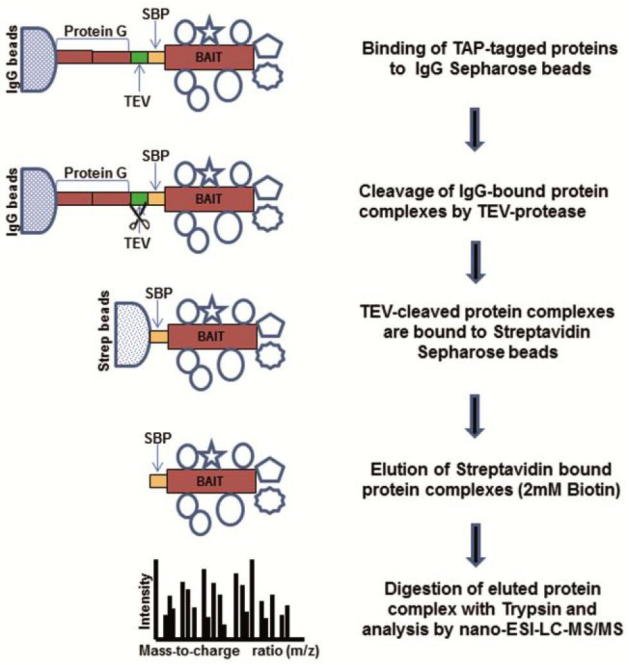

Figure 1 *Illustration of the tandem affinity purification (TAP) method used for isolation of protein complexes from SH-SY5Y cells stably expressing NCL proteins. IgG beads refers to IgG Sepharose beads and Protein G to Protein G domain. Strep beads and SBP are abbreviations for Streptavidin agarose beads and Streptavidin binding peptide, respectively.*

High throughput affinity capture mass spectrometry (AP-MS) experiments have been utilised to analyse protein networks relevant to human disease. A few examples are surveyed below. Proteomic analysis of the human autophagy network in HEK 293 cells, revealed 409 interacting proteins with 751 interactions within the network (Behrends et al., 2010). Autophagy is known to be disrupted in many human diseases, including NCL (Cao et al., 2006; Shacka et al., 2008) . In an *in vivo* proteomic interactome study, brains from the BACHD mouse model that expresses full length human mutant Huntingtin (mHtt) were utilised to identify 747 potential Huntingtin (Htt) interacting partners in distinct mouse brain regions (Shirasaki et al., 2012). Mutations in the *Htt* gene that lead to expansion of the (CAG)n repeats are known to cause Huntington's disease (HD), reviewed in (Goldberg et al., 1994). Recently, the human Hippo interaction proteome was characterized in HEK 293 cells thereby demonstrating 270 interacting partners with 480 protein-protein interactions (PPI) (Hauri et al., 2013). Another study of the human cyclin-dependent kinase [CDK], mitogen-activated protein kinase [MAPK], glycogen synthase kinase [GSK3], CDC-like kinase [CLK]) (CMGC) kinome, utilised HEK 293 cells to identify 652 high-confidence kinase-protein interactions. Cancer-associated proteins (CAPs) were particularly enriched in the CMGC kinase complexes. This work is a potentially important resource for use in studying human pathologies, in which the CMGC kinase complexes maybe perturbed (Varjosalo et al., 2013).

Yeast two-hybrid assays were used to construct interaction networks for ataxias and Huntington's disease (HD) (Kaltenbach et al., 2007; Lim et al., 2006). In the ataxia study, 770 PPI (mostly novel) were detected by a stringent yeast two-hybrid screen and a subset of 62 interactions was validated by co-immunoprecipitation in HEK 293T cells. Several ataxia-causing proteins clustered in a well-connected sub-network and shared many interactors, some of which proved to be ataxia modifying genes in *Drosophila* and mouse models (Lim et al. 2006). HD-centered experiments yielded 234 HD-associated proteins, 104 and 130 identified by yeast two-hybrid and AP-MS (in HEK 293, HeLa, and M17 neuroblastoma cells), respectively. After randomly probing a subset of 60, 45% were found to be genetic modifiers of neurodegeneration in an HD *Drosophila* model (Kaltenbach et al. 2007).

Computational modeling and experimental techniques were employed (Pujana et al., 2007) to identify biomarker genes associated with breast cancer. Several "omic" datasets (human and other organisms) with 118 genes linked by 866 functional associations were used to construct a breast cancer network. Yeast two-hybrid and co-immunoprecipitation assays were applied to the network, thereby extending it and

identifying putative disease genes. This work genetically linked breast cancer susceptibility and centrosome dysfunction.

2.2.3 PPI networks and therapeutic Intervention

Detailed PPI maps of disease genes may clarify disease mechanisms (Pache et al., 2008) and suggest potential points for therapeutic intervention (i.e. drug targets). Interactome maps are robust and therefore well suited for selection of strategic network nodes, in contrast to gene-centreed approaches in which the target biological context is usually ignored. Biological systems, such as disease states, are generally resistant to perturbations and often maintain their functions through various mechanisms (e.g. back-up circuits and fail-safe mechanisms) (Kitano, 2004). Consequently, network positioning (i.e. preference for fewer pathways or topological properties), prioritization of regions critical for driving network traffic and avoidance of back-up circuits that could neutralize drug effects, should all be considered during the selection of new putative drug targets.

Moreover, PPI network based approaches may also further the identification of protein-protein interactions suitable for direct targeting with drug-like compounds (Neduva and Russell, 2006; Wells and McClendon, 2007). This approach presents several advantages over conventional targets (such as enzyme active sites). It offers a more subtle, specific form of regulation that can avoid off-target side effects or total ablation of normal enzyme activity. For instance, Nutlins (a class of drug cancer candidates) were used to block the interaction between tumor suppressor p53 and its negative regulator MDM2, thereby allowing p53 to mediate apoptosis (Vassilev et al., 2004). Several other chemicals have been designed in order to disrupt interactions among translation initiation factors (Moerke et al., 2007) or to sequestrate cytokines, in efforts to impede receptor binding (He et al., 2005). Despite the complexity of identifying small molecules that interfere with specific PPI, new methodologies (Parthasarathi et al., 2008) and technical improvements should provide the necessary toolkit to expand this domain of drug discovery.

2.3 Cells of the central nervous system (CNS)

The brain and spinal cord form the central nervous system (CNS). All multicellular animals (bilateria) with the exception of sponges and jellyfish rely on the CNS to integrate received information and co-ordinate activity to all parts of the body. Our thoughts, actions and emotions all flow through the CNS. Analogous to the computer, the CNS is the motherboard and control panel of the human body. Specific sensory stimuli trigger the CNS to initiate a cascade of physical reactions aimed at interpreting and communicating the signal to the rest of the body, via a network of organs and cells. At a cellular level, the CNS is comprised of neurons and glial cells. Neurons communicate with one another via synapses (Kandel ER, 2000; Purves D, 2011).

2.3.1 Neurons and Glial Cells

A neuron or nerve cell is the fundamental unit of the nervous system. Typically, a human brain comprises of 10^{11} neurons that vary in type and carry-out a multitude of tasks. Similarly to other cells of the human body, neurons comprise of a nucleus, organelles (mitochondria, Golgi), cytoplasm and a cell membrane. However, neurons are unique because they are specialized for electrical signalling due to their extensive branching e.g. arborization of dendrites (Nicholls JG, 2011; Purves D, 2011). Although they no longer regenerate shortly after birth, neurons form connections throughout life. Axons and dendrites are specialized structures of neurons that transmit and receive information, respectively. During communicaton between the nerve cells, connections between neurons (synapses) act as the sites of neurotransmitter release.

Most neurons constitute of three basic parts: a cell body, dendrites, and an axon (Figure 2A). Cell body refers to the bulbous end of a neuron, where the nucleus is located. Dendrites are branched projections that conduct electrochemical stimulation received from other neural cells, to the cell body of the neuron from which they project. An axon is a long, slender projection of a neuron that typically conducts electrical impulses away from the neuron's cell body. The shape of a neuron is mostly determined by its cytoskeleton, which constitutes of microtubules, neurofilaments and actin microfilaments. Asymmetric distribution of organelles within the cytoplasm is facilitated by these filaments (Kandel ER, 2000). Signals are usually transferred from the axon of one neuron to a dendrite of another via a synapse. Neurons are electrically

excitable and maintain voltage gradients across their membranes by coupling metabolically driven ion pumps with membrane ion channels, to generate intracellular-versus-extracellular concentration differences of ions. Changes in the cross-membrane voltage can alter the function of voltage-dependent ion channels. A significant voltage change generates an all-or-none electrochemical pulse (action potential) which travels rapidly along the cell's axon, and activates synaptic connections with other neurons.

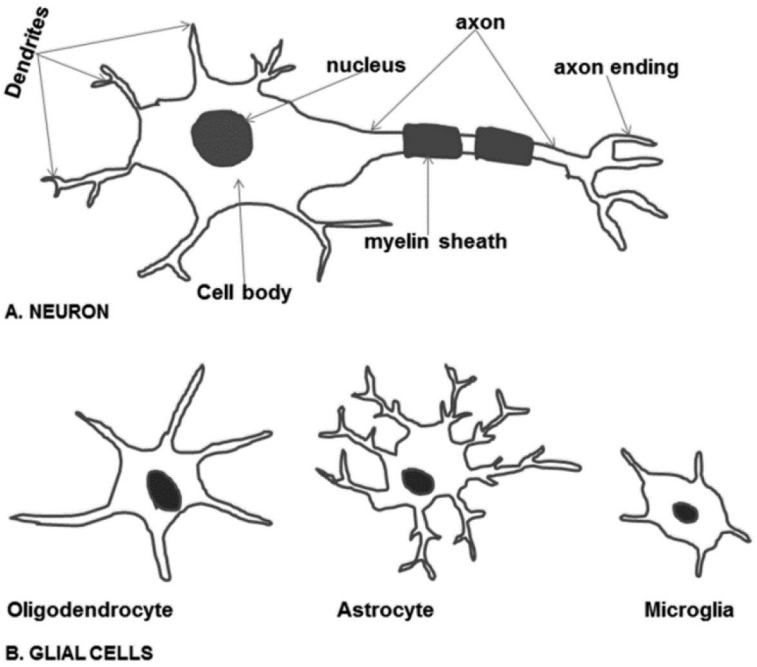

Figure 2 *Cell types of the CNS include: (A) Neurons and (B) Glial cells (oligodendrocytes, astrocytes and microglia).*

Unlike neurons, glial cells do not participate directly in synaptic interactions or electrical signalling, but are involved in supportive roles that define synaptic contacts and maintain neuronal signalling (Purves D, 2011). Glial cells are generally smaller than neurons, lack axons or dendrites and outnumber neurons in the brain by three fold. Known roles of glial cells include: maintenance of the ionic milieu of nerve cells, modulation of nerve signal propagation and synaptic action (control the uptake

of neurotransmitters), providing a scaffold for some aspects of neural development, and aiding in recovery from neural injury.

Three types of glial cells are found in the mature central nervous system (CNS): astrocytes, oligodendrocytes, and microglial cells (Figure 2B). Astrocytes, which are exclusively found in the brain and spinal cord, have a star-like shape derived from their elaborate local processes (Purves D, 2011). They are the most abundant cell of the human brain and have various functions, including: biochemical support of endothelial cells that form the blood-brain barrier, provision of nutrients to the nervous tissue, maintenance of extracellular ion balance, and a role in the repair and scarring process of the brain and spinal cord following traumatic injuries.

Oligodendrocytes are also restricted to the CNS and wrap myelin sheaths around some axons. Myelin sheaths facilitate fast salutatory conduction of action potential by reducing membrane capacitance, while increasing membrane resistance in the internode intervals (Simons and Trotter, 2007). In the peripheral nervous system, the cells that elaborate myelin are called Schwann cells. Microglia constitute 20% of the total glial cell population within the brain (Lawson et al., 1992). They are the resident macrophages of the brain and spinal cord, and thus act as the first and main form of active immune defense in the CNS. Microglia are constantly scavenging the CNS for plaques, damaged neurons and infectious agents (Gehrmann et al., 1995). The brain and spinal cord are considered “immune privileged” organs in that they are separated from the rest of the body by a series of endothelial cells known as the blood-brain barrier, which prevents most infections from reaching the vulnerable nervous tissue.

2.3.2 Synapses

Santiago Ramón y Cajal proposed that neurons are not continuous throughout the body, yet still communicate with each other, an idea known as the neuron doctrine (Lopez-Munoz et al., 2006). A synapse is a point of intersection between the axon and dendrite of a pre- and postsynaptic neuron, respectively (Kandel ER, 2000). Although most synapses have presynaptic sites on their axons, they maybe localised on a dendrite or soma in a few cases. Synapses are found where neurons connect with each other or with muscles and glands. Neural signals are transmitted from the pre- to postsynaptic termini, via a synaptic cleft. The molecular machinery of the presynaptic termini includes: small GTP binding proteins, vesicle-SNARES, calcium and synaptotagmin. Postsynaptic termini comprise of neuroreceptors and target-SNARES

(Kandel ER, 2000). The three basic parts of a synapse include: pre- and postsynaptic termini and a synaptic cleft between the two cells (Figure 3).

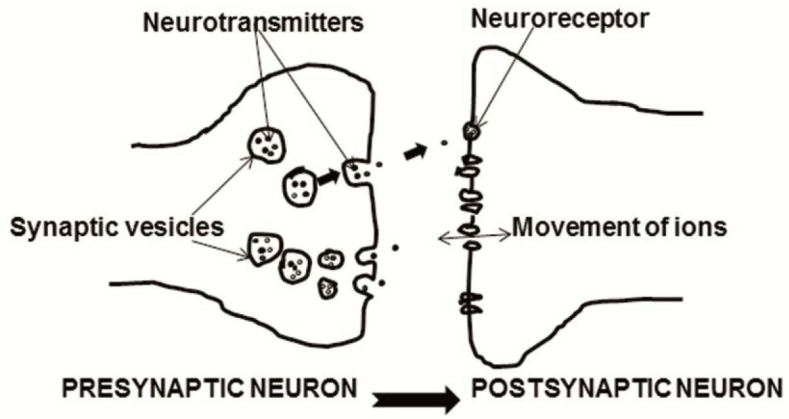

Figure 3 *In a typical synapse, pre- and post- synaptic neurons are separated by a synaptic cleft. The pre-synaptic neuron has synaptic vesicles with neurotransmitters whereas neuroreceptors are found on the post-synaptic neuron.*

Individual neurons form thousands of connections with other neurons via synaptic termini, hence a typical brain has over 100 trillion synapses (Kandel ER, 2000; Nicholls JG, 2011). Functionally related neurons connect to each other to form neural networks (neural nets or assemblies). Astrocytes also exchange information with synaptic neurons, respond to synaptic activity and regulate neurotransmission (Perea et al., 2009). Synapses maybe distinguished as chemical or electrical. In a chemical synapse, the presynaptic neuron releases a neurotransmitter that binds to receptors located in the postsynaptic cell, usually embedded in the plasma membrane (Purves D, 2011). The neurotransmitter may initiate an electrical response or a secondary messenger pathway that may either excite or inhibit the postsynaptic neuron. In an electrical synapse, the presynaptic and postsynaptic cell membranes are connected by special channels called gap junctions that are capable of passing electric current, causing voltage changes in the presynaptic cell to induce voltage changes in the postsynaptic cell. The main advantage of an electrical synapse is the rapid transfer of signals from one cell to the next (Kandel ER, 2000).

2.3.3 Midbrain dopaminergic (DA) neurons and their projections

Nearly 75% of dopaminergic neurons in the adult CNS are found in the ventral midbrain (VM), accounting for 400,000 - 600,000 and 20,000 - 30,000 in the human and mouse brains, respectively (German et al., 1983). Midbrain dopaminergic neurons (DA) arise from the floor plate of the mesenphalon during embryogenesis and develop into three major DA neuron subtypes, namely: A8, A9 and A10 neurons (Ono et al., 2007). A8 neurons which dorsally and caudally extend from A9 neurons form the retrorubal field (RRF) and project to striatal, limbic and cortical areas. Nigral A9 neurons form the substantia nigra pars compacta (SNc) and project to the dorsal striatum along the nigrostriatal pathway, whereas A10 neurons of the ventral tegmental area (VTA) innervate the ventral striatum via the mesocortico-limbic system (Tzschentke and Schmidt, 2000). In Parkinson's disease (PD), the loss of A9 neurons and their striatal projections leads to impaired motor function (Lees et al., 2009; Toulouse and Sullivan, 2008). A9 neurons are critical for the control of voluntary movement, whereas A8 and A10 neurons are involved in the regulation of emotion and reward. The SNc DA (A9) neurons which are known to progressively degenerate in PD, have been observed to be more susceptible to cell death than the A8 and A10 neurons (Alavian et al., 2008; Betarbet et al., 2000). This difference in relative sensitivities of the three dopaminergic neuron groups probably arises from slight variations in their development cues. Alterations in neurotransmission by the mesocortico-limbic dopaminergic pathway are implicated in onset of schizophrenia, drug addiction and depression (Meyer-Lindenberg et al., 2002; Robinson and Berridge, 1993).

Midbrain DA neurons may also be categorized into dorsal and ventral subgroups, on the basis of their morphology and connectivity. The dorsal subgroup (A8, dorsal VTA and SN DA neurons) comprises of round / fusiform shaped, calbindin-positive neurons that express low levels of the DAT transporter and innervate the matrix of the dorsal stiratum, ventral striatal, limbic and cortical areas (Gerfen et al., 1987; Lynd-Balta and Haber, 1994). In contrast, calbindin-negative cells that express higher levels of DAT constitute the ventral subgroup (ventral VTA and SN DA neurons). These more densely packed, angular cells innervate the patch compartment of the striatum and SN pars reticulata (SNr) (Gerfen et al., 1987; Prensa and Parent, 2001).

2.4 Neuronal Ceroid Lipofuscinoses

NCL are the most common inherited progressive encephalopathy of childhood worldwide. Incidence rates vary from 1:67000 in Italy and Germany to 1:12500 in the USA and Scandinavian countries (Santavuori, 1988). The first NCL patients (4 siblings) were described in 1826 by Christian Stengel in Norway. In 1903, F.E. Batten made pioneering clinicopathological studies on several families and later differentiated NCL from Tay-Sachs disease in 1914. Characteristic features of the disease include: early accumulation of auto-fluorescent ceroid-like lipopigment in lysosomes of most cells and degeneration of cortical neurons. Clinically, NCL patients suffer from progressive loss of vision, mental and motor deterioration, epileptic seizures, premature death and dementia, in the rarer adult-onset forms (Haltia, 2006).

Despite progress in NCL genetics, little is known about the physiological functions or interactions of most NCL proteins. From available interaction and genetic data it becomes very clear that system-wide approaches are necessary to reveal all the NCL-related pathways (Jalanko et al., 2006). Bioinformatic analyses and literature searches reveal that several of the NCL proteins can interact with each other, reviewed in (Getty and Pearce, 2011; Kollmann et al., 2013). Similarly to other multifactor gene disorders, NCL proteins may be involved in cross-talk between many cellular pathways and result in similar mechanisms of neurotoxicity. In our recent studies on CLN3-CLN5 and CTSD interactomes, human SH-SY5Y-NTAP-CLN3 / SH-SY5Y-CLN5-CTAP-Puro stable cells and *Ctsd*$^{-/-}$ human / mouse fibroblasts were used as cellular models, respectively (Koch et al., 2013; Scifo et al., 2013). This work pinpointed inter-connections between these NCL proteins with other proteins involved in neurodegeneration, mental retardation and epileptic seizures, as well as functional modules, which can be targeted pharmaceutically.

2.4.1 Genetic basis and disease phenotype correlations of NCL

Genetic linkage analysis and exome sequencing have mostly been utilised to find gene mutations implicated in NCL, reviewed in (Warrier et al., 2013). The human genome sequencing project facilitated the identification of more sequence variants which provided sufficient power for genetic linkage, even with fewer affected families. For instance, the discovery of some genes (*CLN5*, *CLN6*, *CLN7 / MFSD8*, *CLN8*) causative for relatively fewer NCL cases was aided by these technological

advances (Ranta et al., 1999; Savukoski et al., 1998; Siintola et al., 2007; Wheeler et al., 2002). Morever, several mutations in a single gene have been documented to cause different diseases. Examples include: mutations in *CLN14 / KCTD7* which cause three different diseases (Blumkin et al., 2012; Kousi et al., 2012; Staropoli et al., 2012a; Van Bogaert et al., 2007), whereas mutations in *CLN12 / ATP13A2* are implicated in an atypical NCL and Kufor–Rakeb syndrome (Smith et al., 2013). Several mutations in NCL causative genes which are more prevalent in certain populations may be attributed to a founder effect in these regions. An example of this occurence is the 1 kb deletion mutation causing juvenile CLN3 disease cases worldwide. Mutations in NCL causative genes have also been reported to modify disease phenotypes in patients with other gene mutations or diseases. A patient with hetereozygous mutations in *CLN5* and a mutation in *POLGI* exhibited a modified disease profile, with disease onset shortly after birth instead of late infancy (Staropoli et al., 2012b). A genome-wide association study of Ashkenazi Jewish patients with type 1 Gaucher's disease (GD1), revealed *CLN8* as a candidate modifier gene for GD1 that probably functions in sphingolipid sensing or glycosphingolipid trafficking. COSMIC (http://www.sanger.ac.uk/genetics/CGP/cosmic/), which is a comprehensive curated database for somatic mutations in human cancer cells revealed changes in all known NCL genes changes (Zhang et al., 2012).

The NCL mutation database (http://www.ucl.ac.uk/ncl) is a comprehensive resource for known mutations and sequence variations in NCL genes. It has recently been updated to incorporate genetic data of NCL disease patients as reported scientific literature, which should facilitate better correlations between gene changes and disease phenotype. The NCL exhibit both phenotypic convergence and divergence. For instance, clinically similar late infantile variant NCL disease is caused by mutations in *CLN5*, *CLN6*, *MFSD8*, or *CLN8*, reviewed in (Warrier et al., 2013). In contrast, different mutations in *CLN8* cause a mild (Epilepsy with Progressive Mental Retardation, EPMR) or severe (late infantile variant) CLN8 disease (Ranta et al., 1999; Vantaggiato et al., 2009). To date, the mutation in *CLN9* remains unknown whereas most of the mutations in the recently identified NCL causative genes (*CLN11 / GRN, CLN12 and CLN14*) with the exception of *CLN13 / CTSF* were observed in single families (Bras et al., 2012; Smith et al., 2012; Staropoli et al., 2012a). After nearly two decades of research, it is apparent that the molecular genetics of NCL is much more complex than initially predicted.

2.4.2 NCL disease mechanisms

NCL are associated with several disease mechanisms, including: abnormalities in lipid metabolism, myelination; apoptosis; mitochondrial dysfunction and oxidative stress; as well as abnormalities in intracellular metabolism and trafficking, reviewed in (Palmer et al., 2013). Defects in the latter particularly, alterations in autophagy and synaptic vesicle trafficking are explored in detail, below.

Alterations in autophagic pathways are well documented in CLN3, CLN6 and CLN10 diseases. Autophagic vacuoles isolated from *Cln3*$^{\Delta ex7/8}$ knockin mice were observed to have less mature ultrastructural morphology in comparison to those from wild type mice. Microtubule-associated protein 1A / 1B-light chain, 3-phosphatidylethanolamine conjugate (LC3-II) which is an autophagosomal marker was also increased in *Cln3*$^{\Delta ex7/8}$ knockin mice (Cao et al., 2006). Moreover, ATP synthase subunit c was showed to accumulate in cerebellar cells from *Cln3*$^{\Delta ex7/8}$ and *Cln6 / nclf* knockin mice, which suggests a defect in the autophagosome-lysosomal pathway of these NCL (Cao et al., 2011). *Cln10 / Ctsd* knockout mice brains were also reported to have an increased number of autophagic vacuoles and to simultaneously accumulate ATP synthase subunit c (Koike et al., 2005). *Cln6 / nclf* knockin mice displayed age dependent increases in LC3-II and associated generation of neuronal p62 positive aggregates, which is suggestive of disruption in the autophagy-lysosome pathway. It was hypothesized that lysosomal dysfunction during CLN6 deficiency facilitates impairment of constitutive autophagy, which probably enhances neurodegeneration *via* cell toxicity from formed p62 positive aggregates (Thelen et al., 2012).

Synaptic alterations have been observed in most NCL. Electron microscopy studies of cultured neurons from *Cln1 / Ppt1* knockout mice and postmortem brain tissues from an INCL patient, showed that CLN1 deficiency led to reduced synaptic vesicle pools (Kim et al., 2008; Virmani et al., 2005). A possible explanation for the progressive decline in synaptic vesicle pools is that during CLN1 deficiency, palmitoylated synaptic vesicle proteins, such as: synaptotagmin, synaptosomal-associated protein 25 (SNAP-25) and syntaxin 1, remained associated with the membrane (Kim et al., 2008). Reduced synaptic vesicle pools were similarly observed in *Cln6 / nclf* knockout mice, thereby supporting the hypothesis that the presynaptic compartment is re-organised prior to synaptic loss (Kielar et al., 2009). Electron microscopic analysis of *Cln10 / Ctsd* knockout mice brains showed a progressive increase in the number of synaptic vesicles, but a decrease in the frequency of miniature synaptic currents which is suggestive of disruption in the release of

synaptic vesicles (Koch et al., 2011). In recent cysteine-string protein-α (CSPα) knockout mice experiments, SNAP-25 levels and SNARE complex assembly were decreased by 40% and 50%, respectively. The CSPα / CLN4 interaction with SNAP-25 is essential for synaptic vesicle fusion (Sharma et al., 2012).

In addition to impaired intracellular trafficking and endocytosis in CLN1 and CLN3 diseases, there is evidence that synaptic failure and possible defects in recycling of synaptic vesicles constitute part of the pathogenesis of CLN1, CLN4 and CLN10 diseases, reviewed in (Palmer et al., 2013).

2.4.3 NCL proteins and diseases

Nearly 400 mutations in thirteen genes (*CLN1-8, CLN10-14*) are known to cause NCL, with several of them only recently identified (http://www.ucl.ac.uk/ncl). Most human NCL show an autosomal recessive mode of inheritance, and may have variable ages of onset such as congenital, infantile, late infantile, juvenile, adult or even late adult onset according to the severity of mutation. In this study, we focused on four NCL with different ages of onset i.e CLN10 (Congenital NCL), CLN1 (Infantile NCL), CLN3 (Juvenile NCL) and CLN5 (Finnish variant NCL), which are profiled in greater detail in the next sections. NCL phenotypes and their known associated genes are shown in Table 1.

Table 1. Summary list of NCL proteins and functions

NCL-related protein name	*Other names/ synonyms*	*Protein structure and localization*	*Function*
CLN1	palmitoyl protein thioesterase 1 (PPT1)	soluble lysosomal matrix protein	palmitoyl thioesterase
CLN2	tripeptidyl peptidase 1 (TPP1)	soluble lysosomal matrix protein	serine protease
CLN3	-	Late endosomal/ lysosomal membrane protein	unknown, function in vesicular trafficking and fusion postulated
CLN4[80]	cysteine-string protein alpha (CSPα)	cytosolic protein associated to vesicular membranes	Hsc70 co-chaperone, involved in exocytosis
CLN5	-	soluble lysosomal matrix protein	unknown, function in vesicular trafficking postulated
CLN6, CLN4[87]	-	ER-located membrane protein	unknown
CLN7	-	lysosomal membrane protein	Unknown, transmembrane transporter function postulated
CLN8	-	ER/ERGIC-located transmembrane protein	unknown, function in lipid metabolism postulated
CLN9 (postulated)	unknown	-	unknown, function in ceramide synthesis postulated
CLN10	cathepsin D (CTSD)	soluble lysosomal matrix protein	aspartyl endopeptidase
CLN11	progranulin	secreted protein	unknown?
CLN12	ATPase 13A2 (ATP13A2)	lysosomal transmembrane protein	unknown, vATPase, function in regulation of ion homeostasis postulated
CLN13	cathepsin F (CTSF)	soluble lysosomal matrix protein	cysteine protease
CLN14	potassium channel tetramerization domain-containing protein 7 (KCTD7)	cytosolic protein	unknown, BTB/POZ-domain containing protein, function in modulation of ion channel activity postulated

adapted from a review (Kollmann, Uusi-Rauva et al. 2013)

2.4.3.1 CLN10 (CTSD) and Congenital NCL

The *CLN10* gene localised on chromosome 11p15.5 encodes a lysosomal aspartyl protease, cathepsin D (CTSD). Human CLN10 or CTSD is synthesized as a 53 kDa inactive preproenzyme of 412 amino acids, prior to posttranslational modification by glycosylation, mannose 6-phosphate (M6P) residues, and limited proteolysis to yield enzyme isoforms of 47, 31 and 14 kDa, respectively (Gieselmann et al., 1985). Transport of CTSD to lysosomes occurs via M6PR-dependent or -independent pathways (Dittmer et al., 1999; van Meel and Klumperman, 2008), depending on cell type. Amongst several *in vitro* substrates of CTSD is Prosaposin (proSAP), which is cleaved into saposins (A -da D) (Gopalakrishnan et al., 2004) that constitute essential cofactors for hydrolysis of sphingolipids (Kolter and Sandhoff, 2006). *In vivo* substrates of CTSD are yet to be characterized. Besides a role in proteolytic processing, CTSD is also involved in cell proliferation, antigen processing, apoptosis, and regulation of plasma HDL-cholesterol level (Benes et al., 2008; Berchem et al., 2002; Haidar et al., 2006; Moss et al., 2005).

Mutations in the *CLN10* gene are known to cause congenital NCL in human (CLN10, OMIM: 610127) (Fritchie et al., 2009; Siintola et al., 2006) and sheep (Tyynela et al., 2000). Moreover, CTSD has been linked to various neurodegenerative disorders associated with ageing, including: Alzheimer's (Bishop et al., 2008; Nakanishi, 2003; Schuur et al., 2011; Sevlever et al., 2008), Parkinson's (Sevlever et al., 2008) and Creutzfeldt-Jakob (Bishop et al., 2008) diseases. To date, four disease-causing mutations of *CLN10* are known (NCL mutation database www.ucl.ac.uk/ncl/mutation). Ten patients with autosomal recessively inherited congenital NCL were reported, but remain genetically undefined (Siintola et al., 2006). Clinical symptoms of congenital NCL patients include: respiratory insufficiency, rigidity, status epilepticus, and death, within a few weeks after birth. Autopsies of the patients indicated microcephaly with substantial loss of neurons in the cerebral cortex, extensive gliosis, absence of myelin and autofluorescent inclusion bodies with granular osmiophilic deposit, GROD (Steinfeld et al., 2006).

2.4.3.2 CLN1 (PPT1) and Infantile NCL

CLN1 or palmitoyl protein thioesterase 1 (PPT1) is a small 306 amino acid glycoprotein encoded by the *CLN1* gene on chromosome 1p32 (Vesa et al., 1995). The enzyme removes palmitate groups from cysteines of lipid modified proteins

(Camp and Hofmann, 1993). PPT1 is heavily glycosylated and appears as a 37 / 35-kDa doublet in Western blot analysis. Based on the crystal structure of bovine PPT1 (95% homology to the human protein), the enzyme has an α / β-serine hydrolase structure reminiscent of lipases and a catalytic triad constituting of Ser115-His289-Asp233 (Bellizzi et al., 2000). Transport of the overexpressed protein to lysosomes occurs by mannose 6-phosphate receptor (M6PR)-mediated pathway in non-neuronal cells, but is yet to be experimentally validated in neurons (Hellsten et al., 1996; Verkruyse and Hofmann, 1996). Similarly to other posttranslational modifications, palmitoylation is a dynamic process that is utilised by the cell to alter subcellular localization, protein-protein interactions (PPI) or binding capacities of a protein. Palmitoylation is also critical in targeting of proteins for transport to nerve terminals and regulation of trafficking at synapses (Huang and El-Husseini, 2005). Although the *in vivo* substrates and physiological function of PPT1 are unknown, the protein is suggested to be involved in apoptosis (Cho and Dawson, 2000; Zhang et al., 2006), endocytosis (Ahtiainen et al., 2006), vesicular trafficking (Kim et al., 2008), synaptic function (Kielar et al., 2009) and lipid metabolism (Lyly et al., 2008).

Infantile neuronal ceroid lipofuscinosis (INCL, MIM#256730) is caused by mutations in the *CLN1 or PPT1* gene (Vesa et al., 1995). A total of 45 disease causing mutations have been identified for the *CLN1* gene (http://www.ucl.ac.uk/ncl/), including the [R122W] missense mutation that is prevalent in the Finnish population (Das et al., 1998). INCL is apparent after 6 months and is associated with various clinical symptoms, such as: visual failure, microcephaly, seizures, mental / motor deterioration and finally death at 10 years old (Santavuori, 1988). GRODs are present in most cell types of INCL patients (Das et al., 1998).

2.4.3.3 CLN3 and Juvenile NCL

CLN3 is a hydrophobic integral membrane protein comprising of 438 amino acids and encoded by the *CLN3* gene on chromosome 16p12 (Consortium, 1995). The protein has six transmembrane domains with cytoplasmic N- and C-termini (Ezaki et al., 2003; Nugent et al., 2008). CLN3 is differentially glycosylated depending on tissue type (Ezaki et al., 2003; Storch et al., 2007) and in neurons is localised in endosomes/lysosomes, as well as transported to synaptosomes (Kyttala et al., 2004; Luiro et al., 2001; Storch et al., 2007). Although CLN3 has been studied extensively over the past two decades, there is still no consensus on its function. Based on yeast

experiments, CLN3 was proposed to function in endosome-Golgi-retrograde transport (Kama et al., 2011), vacuole protein sorting (Codlin and Mole, 2009), vacuolar pH homeostasis and arginine transport (Kim et al., 2003; Padilla-Lopez and Pearce, 2006). Involvement of CLN3 in intracellular trafficking (Luiro et al., 2004; Metcalf et al., 2008; Uusi-Rauva et al., 2012), lipid metabolism (Hobert and Dawson, 2007; Narayan et al., 2006), galactosyl-ceramide transport (Rusyn et al., 2008), sphingolipid homeostasis (Rusyn et al., 2008), autophagy (Behrends et al., 2010; Cao et al., 2006), lysosomal arginine transport (Ramirez-Montealegre and Pearce, 2005), pH homeostasis (Golabek et al., 2000), and apoptosis (Lane et al., 1996), was demonstrated in mammalian cells and mouse models.

Juvenile neuronal ceroid lipofuscinosis (JNCL / CLN3 disease; MIM#204200) (Consortium, 1995) is caused by mutations in the *CLN3* gene. Globally, JNCL is the most common NCL with onset between 4 and 10 years of age (Consortium, 1995). Thus far, 57 *CLN3* gene mutations (including 20% with a JNCL phenotype) are known (http://www.ucl.ac.uk/ncl). The most prevalent *CLN3* gene mutation is the 1.02 kb deletion carried by 73% and 90% of the CLN3 disease patients worldwide and in Finland, respectively (Consortium, 1995; Jarvela et al., 1996). Clinical symptoms of JNCL include: progressive loss of vision starting at 5-10 years of age, mental deterioration, followed by epileptic seizures, loss of motor skills and death by the third decade (Haltia, 2006). JNCL patients may also display various psychiatric symptoms, such as aggressiveness, depression and sleep deficits (Williams et al., 2006). Autopsy examination of the CLN3 disease brains shows a narrow cerebral cortex and decreased brain weight. The presence of vacuolated lymphocytes is a distinguishing feature of JNCL (Mole et al., 2005).

2.4.3.4 CLN5 and Finnish variant LINCL

Mutations in *CLN5* are implicated in Finnish variant late infantile NCL ($vLINCL_{Fin}$). The function of the soluble 407 amino acid CLN5 protein is unclear. CLN5 isoforms of various molecular weights (46.3, 43.4, 41.5, and 40.3 kDa) are generated from the four (Met-1, Met-30, Met-50, and Met-62) in-frame alternative initiator codons, respectively (Savukoski et al., 1998). Similarly to other NCL, CLN5 is also heavily glycosylated and so is expected to migrate at higher molecular weights (60-80 kDa), relative to its predicted size (Isosomppi et al., 2002; Vesa et al., 2002). Experiments with mouse CLN5 have suggested trafficking of the protein to lysosomes via the mannose-6-phosphate receptor (MPR) pathway, however alternative trafficking

routes to the lysosomes have also been demonstrated in MPR-deficient fibroblasts (Holmberg et al., 2004; Schmiedt et al., 2010; Sleat et al., 2006).

CLN5 is mostly localised to lysosomes (Bessa et al., 2006; Isosomppi et al., 2002; Schmiedt et al., 2010), however, pathogenic mutations may cause the protein to be retained in the ER / Golgi (Isosomppi et al., 2002; Lebrun et al., 2009; Schmiedt et al., 2010). Mutated CLN5 proteins are known to be glycosylated, which suggests that they are trafficked from the ER to the Golgi apparatus, where they are glycosylated (Vesa et al., 2002). CLN5 is synthesized as a preprotein from any of the four initiator codons (depending on cell type / condition), followed by cleavage of its N-terminal signal peptide in the ER. Mannose-type sugars are then attached to the protein, prior to its trafficking to the Golgi apparatus for more glycosylation and maturation to a 50 kDa protein. The mature CLN5 is trafficked to the lysosomes either through the MPR or secretory pathway (Schmiedt et al., 2010).

Although CLN5 has been suggested to interact with other NCL proteins (Vesa et al., 2002), our investigations of CLN5 interacting partners (IP) using a TAP-MS strategy in SH-SY5Y stable cells did not yield any NCL proteins. A possible explanation for the discrepancy is that previous CLN5 studies relied on overexpression of the protein in COS-1 or BHK cells, which probably fails to account for important posttranslational modifications critical for the protein (Isosomppi et al., 2002; Lebrun et al., 2009; Vesa et al., 2002). CLN5 probably has an important unknown function because loss of the protein results in CLN5 disease (vLINCL$_{Fin}$). Recent experiments using CLN5 depleted HeLa cells suggest a role for CLN5 as an endosomal switch for recycling of lysosome sorting receptors to the Golgi apparatus, thus enabling vesicular trafficking and cargo sorting through its recruitment / activation of Rab7 and a retromer (Mamo et al., 2012).

2.4.4 Mouse models of NCL

Mouse models of NCL share a similar phenotype with the human disorder, including widespread regional atrophy and significant loss of GABAergic interneurons in the hippocampus and cortex (Cooper et al., 2006; Mitchison et al., 2004). As such, the various available NCL mouse models (Table 2) are relevant for understanding the pathology and pathophysiology of the disease. Moreover, due to the severity of NCL, therapeutic strategies to combat the disease need to be first established in mouse models before translation to humans.

Table 2. Mouse models of NCL with identified human mutations

Mouse model	*Mutation*	*Onset of Neurological symptoms*	*Mortality*
Ctsd knockout	Replacement insertion of neo cassette in exon 4	3 weeks	26 ± 1 days
Ppt1 (exon 9) knockout	Replacement insertion of neo cassette in exon 9	≥ 2 months	216 days
$Ppt1^{\Delta ex4}$ knockout	Cre / lox-mediated deletion of exon 4		200 days
Tpp1 neo^{ins}-*Arg446His* knockout	Replacement insertion of neo cassette in intron 11+ Arg446His mutation of exon 11	≥ 7 weeks	132–155 days, strain dependent
Tpp1 Arg446His hypomorphs	Cre / lox-mediated removal of neo cassette from Tpp1 neoins-Arg446His knockout	≥ 9 months	Median 603 days
Cln5 knockout	Replacement insertion of neo cassette in exon 3	≥ 13 weeks	unknown
$Cln6^{nclf}$ spontaneous mutant	Single bp insertion & resultant frame shift mutation of exon 4	≥ 9 months	12 months
$Cln8^{mnd}$ spontaneous mutant	Single bp insertion & resultant frame shift mutation	≥ 2–3 months	10–12 months
Cln3 knockout	Replacement insertion of neo cassette in exons 1–6	≥ 3 months	unknown
Cln3 knockout	Replacement insertion of neo cassette in exons 7–8		
$Cln3^{\Delta ex7/8}$ knockin	Cre / lox-mediated deletion of exons 7–8 and neo cassette		
$Cln3^{lacZ/lacZ}$ knockin	5' β-galactosidase reporter construct followed by deletion of majority of exon 1 and all of exons 2–8	≥ 2 months	unknown

adapted from a review (Shacka 2012)

2.4.4.1 *Ctsd* knockout mouse model of congenital NCL

The *Ctsd* knockout (*Ctsd*$^{-/-}$) mouse model for congenital NCL was created by targeted disruption of the *Ctsd* gene in the open reading frame of exon 4, which abolished CTSD protein levels and enzyme activity (Saftig et al., 1995). Although *Ctsd*$^{-/-}$ mice appear normal during the first two weeks of life, they progressively and rapidly lose weight, exhibit severe intestinal necrosis, thromboembolia, lymphopenia and finally die by postnatal day 26 ±1(Saftig et al., 1995). Similarly to human patients of congenital NCL, *Ctsd* knockout mice are also characterized by ultrastructural appearance of GRODs (Mole et al., 2010). Robust autofluorescence and ultrastructural appearance of lamellar structures are observed in both human congenital NCL and *Ctsd*$^{-/-}$ mouse brains (Koike et al., 2000; Shacka et al., 2007). The predominant lysosomal storage product in *Ctsd* knockout mice is subunit c of mitochondrial ATP synthase (SCMAS) (Shacka et al., 2007). Ultrastructural and Western blot analyses show that *Ctsd*-deficient mouse brains dramatically accumulate autophagic vacuoles adjacent to the GROD (Koike et al., 2000; Koike et al., 2005; Shacka et al., 2008; Walls et al., 2007)

2.4.4.2 *Ppt1* (exon 9) and *Ppt1*$^{\Delta ex4}$ knockouts: mouse models of Infantile NCL

There are two different mouse models of Infantile NCL (INCL): *Ppt1* (exon 9) and *Ppt1*$^{\Delta ex4}$ knockouts. The *Ppt1* (exon 9) knockout was created by inserting a neo cassette into exon 9 of the *Ppt1* gene (Gupta et al., 2001); and the *Ppt1*$^{\Delta ex4}$ model involved Cre / lox-mediated deletion of exon 4 (Jalanko et al., 2005). PPT1 activity and protein levels were abolished in both mutations (Gupta et al., 2001; Jalanko et al., 2005). GRODs were observed in *Ppt1*-deficient mice brains (Galvin et al., 2008; Gupta et al., 2001; Jalanko et al., 2005), and an increase in saposins A & D was shown in neurons or fibroblasts derived from *Ppt1*$^{\Delta ex4}$ mice (Ahtiainen et al., 2007). These characteristic pathological features of *Ppt1*-deficient mice closely mimic human INCL (Mole et al., 2010; Tyynela et al., 1993). *Ppt1*$^{\Delta ex4}$ and *Ppt1*-exon 9 mice undergo progressive weight loss and have a shortened lifespan of about 200 and 216 days, respectively (Gupta et al., 2001; Jalanko et al., 2005).

Neurologically, *Ppt1*-deficient mice display a progressive onset of seizures, abnormal clasping behavior, loss of motor coordination and vision loss, which are collectively suggestive of a sudden loss of brain mass (Bible et al., 2004; Jalanko et

al., 2005; Macauley et al., 2009). Dramatic neuron loss has been observed in the thalamus, cortex, hippocampus (both pyramidal neurons and GABAergic interneurons) and cerebellum (Purkinje- early and granule cells- later) by 6-7 months (Gupta et al., 2001; Jalanko et al., 2005; Kielar et al., 2009).

2.4.4.3 *Cln5* knockout mouse model of Finnish variant LINCL

The *Cln5* knockout mouse model of Finnish variant LINCL ($vLINCL_{Fin}$), was created by inserting a neomycin cassette into exon 3 of the C*ln5* gene, thus resulting in a frame shift mutation that introduces a premature stop codon, and leads to a truncated protein (Kopra et al., 2004). Similarly to features of human $vLINCL_{Fin}$, *Cln5*-deficient mice also display progressive thamalocortical accumulation of autofluorescence, as well as ultrastructural detection fingerprint and curvilinear profiles (Kopra et al., 2004; Mole et al., 2010). Although the lifespan of *Cln5*-deficient mice is yet to be established, the phenotype of these mice has been observed even at 12 months of age (von Schantz et al., 2009). In general, brain pathology of *Cln5*-deficient mice is milder than other mouse models of NCL mutations and mimics the mild severity of the Finnish population harboring *CLN5* mutations (Kopra et al., 2004). *Cln5* mutant mice exhibit a late-onset, brain-region specific atrophy that is characterized by early (4 months) and progressive cortical neuron loss and synaptic pathology, which precedes neuron loss of thalamic nuclei at 12 months (von Schantz et al., 2009). Loss of GABAergic interneurons has also been documented throughout the *Cln5*-mutant mouse brain in addition to a progressive inflammatory component (astrocytosis and microglial activation at 4 and 12 months, respectively) (Kopra et al., 2004; von Schantz et al., 2009).

2.4.4.4 *Cln3* knockout and knockin mouse models of juvenile NCL

To date, four distinct *Cln3* mouse models of juvenile NCL (JNCL) have been generated: two knockout (Greene et al., 1999; Katz et al., 1999; Mitchison et al., 1999) and knockin mice (Cotman et al., 2002; Eliason et al., 2007). *Cln3* knockout mice resulted from replacement of either exons 1–6 (Greene et al., 1999; Katz et al., 1999; Mitchison et al., 1999) or exons 7–8 (Katz et al., 1999) of *Cln3* with a *neomycin* cassette, thereby disrupting the gene. The first *Cln3* knockin mouse was generated by a Cre/lox approach (Cotman et al., 2002) that closely mimics the ~1kb

deletion in human JNCL. Exons 7 and 8 (~1kb) of the *Cln3* gene were replaced by "knocking-in" a "floxed" *PGKneo* cassette via homologous recombination (Cotman et al., 2002). In the *Cln3* knockin reporter mouse, the β-galactosidase (β-gal) gene was "knocked-in" to replace most of exon 1 and exons 2–8 (Ding et al., 2011; Eliason et al., 2007).

Each of these mice exhibits abundant autofluorescent storage material and the ultrastructural appearance of fingerprint profiles. The major storage product of JNCL, SCMAS, has also been documented in *Cln3* mouse models (Cao et al., 2011; Fossale et al., 2004; Mitchison et al., 1999). *Cln3* mutant mice display a delayed but progressive onset of neurological deficits (⩾3 months; visual; learning & memory; motor dysfunction) that correlate with noticeable neuron loss in several brain regions including the retina, thalamus, cortex, cerebellum, substantia nigra and striatum (Cotman et al., 2002; Pontikis et al., 2005; Weimer et al., 2007).

2.4.4.5 Other NCL mouse models

Two mouse models of Tripeptidyl peptidase 1 (*Tpp1)* deficiency are available. A *Tpp1* neo^{ins}-Arg446His knockout was created by inserting a neo cassette into intron 11 of *Tpp1*, combined with a missense Arg446His mutation in exon 11 (Sleat et al., 2004). The second mouse model of *Tpp1* (*Tpp1* Arg446His hypomorphs) involves Cre-mediated excision of the neo cassette, which allows for transcription of full length TPP1 mRNA with the missense mutation (Sleat et al., 2008). In the first mouse model (neo^{ins}-Arg446His), TPP1 activity is undetectable (Sleat et al., 2004; Sondhi et al., 2007), whereas the second one has varying amounts of residual enzymatic activity that correlate with progression of the classic late-Infantile NCL (cLINCL) phenotype in these mice. *Tpp1*-deficient mouse brains accumulate SCMAS storage product (Sleat et al., 2008), as is also observed in human classic LINCL (Ezaki et al., 2000; Goebel et al., 1979).

The *Cln6^{nclf}* spontaneous mutant model of vLINCL was mapped to the same chromosome as *Cln6*, the gene linked to vLINCL in families of Indian ancestry and Costa Ricans descended from Spain (Gao et al., 2002). The NCL-like phenotype resembles many aspects of human NCL, hence the acronym "nclf" for "neuronal ceroid lipofuscinoses" (Bronson et al., 1998). The *Cln6^{nclf}* mutation was confirmed as a single bp insertion in exon 4, resulting in a frame shift mutation and a novel 36 kDa gene product, similar to that of human vLINCL (Gao et al., 2002). *Cln6^{nclf}* homozygotes exhibit progressive retinal atrophy and paralysis by 9 months, a

phenotype similar to that of the *Cln8*^{mnd} mouse, which also harbors a spontaneous mutation (Bronson et al., 1998). Brains of *Cln6*^{nclf} mice accumulate luxol fast blue stain for lipoprotein in the cytoplasm of cortical cells, and also exhibit ultrastructural appearance of inclusions with curvilinear, fingerprint profiles and laminar structures (Bronson et al., 1998), all of which are neuropathological features of vLINCL (Mole et al., 2010).

The *Cln8*^{mnd} spontaneous mutant mouse model of variant vLINCL is characterized by progressive loss of motor activity in homozygotes. It was originally thought to represent a variant model of ALS, hence the acronym "mnd" for "motor neuron disease" (Bronson et al., 1993). A single bp insertion in the *Cln8* gene that results in a frame shift mutation and a severely truncated protein was confirmed to be the gene mutation in *Cln8*^{mnd} mice. Severity of the mouse model phenotype resembles the one for a subset of Turkish EPMR / CLN8 patients (Ranta et al., 1999). *Cln8*^{mnd} mice have similar pathology to the human NCL, including ultrastructural detection of curvilinear profiles and storage of autofluorescent cytoplasmic inclusions, which are especially pronounced in the hippocampus, cortex and thalamus (Cooper et al., 1999; Pardo et al., 1994; Rodman et al., 1998). GABAergic neuron and interneuron populations have been shown to accumulate storage material prior to neuron loss (Cooper et al., 1999).

2.5 Therapeutics of NCL

Therapies for treatment of NCL should include small molecules able to cross the blood brain barrier (BBB) and hinder neurodegeneration or repair its effects, thereby restoring proper function of the CNS (Hobert and Dawson, 2006; Pierret et al., 2008). Enzyme replacement therapy, gene therapy and pharmacological intervention are profiled below.

2.5.1 Enzyme Replacement therapy

A strategy to produce and deliver enzymes in the brain was devised by Chen *et al.*, who utilised epitope-modified adeno-associated viral vectors (AAVs) to express enzymes deficient in lysosomal storage disorder (LSD) mice. Enzyme activity was reconstituted throughout the brain and disease phenotypes of β-glucuronidase and

Tpp1-deficient mouse models were improved (Chen et al., 2009). Enzyme replacement therapy (ERT) approaches are well suited for the delivery of soluble NCL proteins (CLN1, CLN2 and CLN10) and require global reconstitution of deficient enzyme activity throughout the brain.

Recombinant CLN2 or TPP1 was successfully overexpressed in CHO cells and correctly targeted to lysosomes (Lin and Lobel, 2001). Following enzymatic treatment, *Tpp1*-deficient mice showed dramatically reduced SCMAS, improved neuropathological profile i.e. decreased: resting tremor, gliosis in the motor cortex, autofluorescence and finally, a partial rescue of deep cerebellar nuclei (Sleat et al., 2008). Recombinant human CLN1 or PPT1 was also similarly processed and tested, as in the CLN2 study (above). Intravenous injection of the human CLN1 to PPT1-deficient mice resulted in widespread distribution of the enzyme to peripheral organs and only minimal delivery to the brain. ERT is a potentially worthy treatment for NCL, provided that improvements in its effectiveness may be achieved by intraventricular infusions, chemical modifications or chronic high-dose therapies.

This therapy is hindered by the risk of developing antibodies against the intravenously delivered enzymes, an occurrence that would inactivate their activity (Wang et al., 2008). Moreover, the delivery of enzymes to the brain is an inefficient strategy for treatment of CNS pathologies, because of the blood brain barrier (Haskins, 2009). Another drawback of ERT is that it cannot mimic the function of transmembrane proteins which account for most NCL proteins (Pierret et al., 2008).

2.5.2 Gene Therapy

Delivery of functional genes to the brain involves intracranial injection of viral vectors that express deficient gene products. This approach was successfully demonstrated in CLN1 and CLN2 patients, where storage material could be removed thereby rescuing the cells. Preclinical studies were conducted in *Cln2*$^{-/-}$ mice, in which AAV2 and AAV5 vectors carrying CLN2 were introduced and shown to locally express high levels of TPP1 for at least 18 months. Consequently, storage granules in the CNS of these mice were cleared but no improvement in performance or mortality was observed (Passini et al., 2006; Sondhi et al., 2007).

Following the preclinical experiments, a Phase I trial to test the safety of gene transfer vectors in CLN2 disease patients was initiated by R. Crystal and collaborators (Worgall et al., 2008). Recently, neonatal *Cln3*$^{\Delta ex7/8}$ mice administered with a serotype rh.10 adeno-associated virus vector expressing the human CLN3

(AAVrh.10hCLN3), were partially rescued for neurological lysosomal storage pathology and astrocytosis (Evans et al., 2013). Neurotropic gene delivery with expression of the proteins at therapeutic levels over a long duration, may offer the best chance for successful gene therapy in combating NCL and other LSD that affect the CNS (Haskell et al., 2003; Sondhi et al., 2007).

2.5.3 Pharmacological Intervention

The glutamatergic system which plays a prominent role in synaptic plasticity, learning, and memory, is also a potent neuronal excitotoxin during pathology (Zarate and Manji, 2008). Excitatory neurotransmission that mediates the fast desensitizing excitation of many synapses is partly dependent on functioning of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors. Consequently, AMPA receptor inhibitors e.g. [+/−]-7-acetyl-5-[4-aminophenyl]-7, 8-dihydro-8-cyano-8-methyl-9H-1, 3-dioxolo-[4,5-h]-2, 3-benzodiazepine (EGIS-8332) may potentially provide therapeutic relief to CLN3 disease patients (Kovacs and Pearce, 2008; Kovacs et al., 2006). *Cln3*-deficient mice were observed to have elevated levels of glutamate within the CNS, and exhibited selective sensitivity of their cerebellar granule cells to AMPA glutamate receptor overactivation (Kovacs et al., 2006). The latter observation suggested that CLN3 deficiency may lead to cerebellar dysfunction via its effects on the function of AMPA receptors (Kovacs et al., 2006). Attenuation of AMPA receptor function with low doses of EGIS-8332 resulted in inhibition of AMPA receptor activity and substantial improvement in motor skills.

A combination of Cystagon and N-acetylcysteine (Mucomyst) was tested in a clinical trial as a treatment for children inflicted with classic infantile CLN1 disease, reviewed in (Kohan et al., 2011). Results from Phase II showed favorable anti-apoptotic properties, reduced ceroid lipofuscin aggregates and diminished progression of neurological symptoms. Administration of an alpha adrenoceptor beta2-agonist (Clenbuterol racemate), boosted axon regeneration lesions of motor neurons in the *mnd* mouse. The mice were also observed to have improved survival and maintenance of functional motor neurons (Zeman et al., 2004).

3 Aims of the study

NCL are genetically heterogeneous yet share a uniform neuropathological profile. We therefore aimed to apply systematic approaches including: functional proteomics, bioinformatics, and mouse disease models, towards analysis of disease pathways associated with NCL in the brain. Despite extensive studies over the past two decades, the physiological functions and interactions of most NCL proteins remain unresolved.

The goal of this thesis work was to contribute towards addressing both shortcomings. In order to have a representative spectrum of NCL, we focused on four disease genes with different ages of onset: *CLN10* (congenital), *CLN1* (infantile classic, late infantile, juvenile, adult), *CLN3* (juvenile, classic) and *CLN5* (late infantile variant, juvenile, adult).

The following specific aims were addressed:

(i) Systematic characterization of the *Ctsd*$^{-/-}$ / *Cln10*$^{-/-}$ mice synaptic proteome, to unravel mechanisms underlying cathepsin D ientiency by quantitative proteomics.

(ii) Mapping the CLN3-CLN5 protein interactome in SH-SY5Y human neuroblastoma cells by label-free quantitative proteomics.

(iii) Characterization of the human CLN1 (PPT1) interactome in SH-SY5Y human neuroblastoma cells by label-free quantitative proteomics.

4 Materials and Methods

Materials and methods used in this study are summarised in Table 3 below. They are referred to by roman numerals indicating the original publication in which they were used. Detailed descriptions of the methods are presented in the following text and in the original publications

Table 3. Summary of experimental methods used in this study

Experimental methods	***Used in publication***
Isolation of synaptosomal fractions	I
Quantitative proteomics analyses (iTRAQ labelling, nano-LC/ESI/MS/MS)	I
Mammalian cell culturing	I, II, III
Immunofluorescence microscopy	I, II, III
Immunohistochemistry	I, III
Quantitative Western blot anaysis	I
Western blot anaysis	II, III
In vitro wound healing assay	I
Co-immunoprecipitation	I, II, III
Gateway recombinant cloning	II, III
Generation of SH-SY5Y human neuroblastoma cells stably expressing NCL	II, III
Retroviral infections	II, III
Tandem/ Single step affinity purification	II / III
Label free Quantitative proteomics analyses (nano-LC/ESI/MS/MS, SAINT analysis)	II, III
Label free Quantitative proteomics analyses (nano-LC/ESI/MS/MS, TransOmics analysis)	III
Filter assisted sample preparation (FASP)	III
Bioinformatic analyses (MGI, NCBI, UniHI,GeneMania, Human Gene Atlas, DAVID, PID and OMIM)	I
Bioinformatic analyses (DAVID, Cytoscape, OMIM, Corum, Hippie and FunCoup)	II
Bioinformatic analyses (Cytoscape, ClueGO, CSSPalm 4.0, OMIM and FunCoup)	III

4.1 DNA expression constructs (II, III)

Full length human entry clones were shuttled in TAP-tagged vectors and utilised for TAP-MS, AP-MS, co-immunoprecipitation, as well as immunofluorescence confocal microscopy experiments. CLN3 (IOH3475) and CLN5 (OCABo5050F1210) entry clones purchased from Source BioScience UK Limited (Nottingham, UK), were shuttled in pCeMM-NTAP(GS)-Gw (NTAP) (Burckstummer et al., 2006) and pES-CTAP-Puro (CTAP-Puro) (Scifo et al., 2013), respectively, prior to use in TAP-MS experiments. The PPT1 (CCSB 5732) entry clone that was also shuttled in pES-CTAP-Puro and used in AP-MS experiments, was a kind gift from Prof. Erich Wanker (MDC, Berlin). Recombination of DNA fragments was performed using the LR clonase reaction (Life Technologies Europe BV, Espoo, Finland) and analysed with BsrGI restriction enzyme. Entry clones used for co-immunoprecipitation and immunofluorescence confocal microscopy experiments, included: CLN3 (IOH3475), DBH (OCAAo5051B0535D), DPM1 (IOH7445) and SEC61A1 (OCABo5050C1119D), purchased from Source BioScience UK Limited; SLC25A10 (RZPDo839F08152), SLC25A11 (RZPDo839G0296) and SLC25A22 (RZPDo839E0876), which were kind gifts from Prof. Erich Wanker (MDC, Berlin). They were shuttled into pcDNA3.1/nV5-Dest (Life Technologies Europe BV), pcDNA3.1-ProtA-D57 (E. Wanker) Gateway vectors and similarly processed as indicated above.

4.2 Retroviral production, transduction and Stable cells (II, III)

Retroviral particles were generated from HEK 293T cells into which retroviral vector DNA was simultaneously introduced (DuBridge et al., 1987; Pear et al., 1993) with two packaging plasmids: pCMV-Gag-Pol vector (Sharma et al., 1997) and pVSV-G (Naldini et al., 1996). The three plasmids were introduced into the cells via the calcium phosphate method as suggested by the manufacturer (Life Technologies Europe BV), at ratios of 7.5:5:1, respectively. Low passage (P5-10) SH-SY5Y cells were infected with cell-free virus, that was processed by harvesting at two days post transfection and filtration through 0.45 μm pore size filters (Millex-HV Filter Unit, Millipore, Ireland Ltd). DMEM: F12 Ham's media (1:1), supplemented with Penicillin (100 μg/ml), Streptomycin (100 μg/ml), Glutamine, non-essential amino acids (1x) and 10% FBS (Life Technologies Europe BV), was used to grow the cells

at 37°C under humidified atmosphere of 95% air and 5% CO_2. We selected stable cells by either sorting for GFP positive cells (NTAP-tagged) or expanding surviving cells after puromycin selection (CTAP-Puro tagged). Further validation of the stable cells was performed by immunocytochemistry and Western blot analysis. We finally maintained the stable cells in an undifferentiated state ($\leqslant$ 80% confluence) and periodiocally checked their growth rates and morphological features.

4.3 Cell culturing and Co-immunoprecipitation (I, II)

DMEM, supplemented with Penicillin (100 µg/ml), Streptomycin (100 µg/ml), Glutamine and 10% FBS was utilised to grow fibroblasts (I) or HEK 293 cells (II) at 37°C, under humidified atmosphere of 95% air and 5% CO_2. Pairs of V5-tagged IP and PA- tagged bait constructs (or vice versa) were transiently co-transfected in HEK-293 cells in 24 well plate format, using Fugene HD transfection reagent (Roche Diagnostics Oy, Espoo, Finland) according to the manufacturer's instructions. Transiently transfected cells were harvested after 48 h, with lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM DTT and 10% glycerol) supplemented with benzonase (E1014, 250U) and protease inhibitors: 1 mM PMSF (P7626; Sigma-Aldrich Finland Oy, Helsinki, Finland) and 1 x protease inhibitor cocktail (Cat. No. 04693116001, Roche Diagnostics Oy). Cell lysis was performed for 30 min. All handling and processing of samples was done at 4°C. Cytoplasmic extracts were subjected to SDS-PAGE and checked for uniform gene expression profiles. Isolation of protein complexes was achieved by incubating 100 µl of the cytoplasmic extractswith 10 µl Dynabeads M-280 Sheep anti-Rabbit IgG (11204D; Life Technologies Europe BV) and shaking for 1 h. Bound The beads were washed three times with 100 µl minimum lysis buffer, in order to minimise unspecific interactions followed by resuspension equal volumes (25 µl) of PBS and 2X sample loading buffer. Co-immunoprecipitation with CLN3 (II) was probed with mouse monoclonal anti-V5 (R960-25; 1:5,000, Life Technologies Europe BV).

4.4 Western blotting and Antibodies (I, II, III)

The following primary antibodies were utilised: mouse monoclonal anti-Myelin PLP [plpc 1] (ab9311, 1:2000), rabbit polyclonal anti-MAP2 (ab24640-50, 1:2000), rabbit polyclonal anti-CLN3 (ab75959, 1:700), mouse monoclonal anti-Myc [9E10] (ab32, 1:1000) and mouse monoclonal anti-LAMP 1 [H4A3] (ab25630, 1:300) (Abcam plc, Cambridge, UK); rabbit polyclonal anti-MBP (1:1000) (Dako, Cambridge, UK); rabbit polyclonal anti-CNPase (2986, 1:2000) and rabbit monoclonal anti-Gapdh (14C10, 1:2000) (Cell Signalling); rabbit polyclonal anti-human-PPT1 (1:500) (Hellsten et al., 1996); rabbit polyclonal anti-CLN5 [C/32] (1:500) (Schmiedt et al., 2010); mouse monoclonal anti-V5 (R960-25, 1:5,000) and mouse monoclonal anti-ATP5B [clone 3D5AB1] (A21351, 1:1000) (Life Technologies Europe BV); rabbit polyclonal anti-PA (5500-100; 1:10,000, Biovision, Germany); mouse monoclonal anti-VCP [#612182] (1:1000) (BD Transduction Laboratories^{TM}); rabbit polyclonal anti-DBH (NBP1-31386, 1:1000) (Novus Biologicals, Cambridge, UK); mouse monoclonal anti acetylated tubulin (T6793, clone 6-11B-1, 1:5000) and mouse monoclonal anti-β-actin [AC15] (A1978, 1:1000) (Sigma-Aldrich Finland Oy); anti-β-actin [AC15] (sc-69879; 1:1000) (Santa Cruz, US); mouse monoclonal anti-FAK (clone 2A7, 2–4 μg/ml) and mouse monoclonal anti-PY20 (2–4 μg/ml) (Upstate-Millipore); mouse monoclonal anti-VII F9 (clone F9B11B4, 2–4 μg/ml) (Glukhova et al., 1990); mouse monoclonal anti-β1-integrin subunit (102DF5, 2–4 μg/ml) (Ylanne and Virtanen, 1989); mouse monoclonal anti-talin (clone TA205, 2–4 μg/ml) (Serotec, Oxford UK); and rat monoclonal anti- α5-integrin subunit (BIE5, 1:5) (Werb et al., 1989). Each antibody was used according to the manufacturer's protocols.

4.5 Tandem Affinity Purification (II, III)

SH-SY5Y cells stably expressing human NCL proteins (NTAP-CLN3, CLN5-CTAP-Puro or PPT1-CTAP-Puro) were grown as monolayer cells to 80% confluency and harvested for affinity purification experiments. Five 150 mm plates (1×10^8 cells) of the cells were utilised for each experiment. After washing cells three times with ice cold 1x PBS, they were flash frozen prior to lysis. Cells were lysed in 5 ml Lysis buffer (50 mm HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 10% glycerol) supplemented with benzonase nuclease (250 U, E1014) and protease

inhibitors (1 mM PMSF and 1x protease inhibitor cocktail), for 30 min at 4°C. Cytoplasmic extracts were obtained from the cell lysates by centrifugation at maximum speed (18,000 rpm) for 10 min. Batch purification of the cytoplasmic extracts were performed by incubation on 200 µl of packed IgG-Sepharose 6 Fast Flow resin (Amersham Biosciences) for 3 h at 4°C. The bound resin was washed three times with 500 µl of 1x TBS-MNGZ (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2.5 mM $MgCl_2$, 0.1% NP-40, 10% glycerol) and TEV cleavage buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 1 mM DTT). Cleavage of tagged proteins from the beads was achieved with 200 or 100 units of TEV protease (Life Technologies Europe BV) in a 300 µl reaction performed for 16 h or 4 h, at 4 °C or 37 °C, respectively. Elution of TEV protease cleaved protein complexes was donewith 500 µl of 1x TBS-MNGZ. TEV eluates were subsequently incubated with 200 µl of packed Streptavidin beads (Sigma-Aldrich Finland Oy) for 4 h at 4 °C. Streptavidin resin bound with TEV eluate was washed three times with 500 µl of 1x TBS-MNGZ and competitively eluted with 2.5 mM Biotin diluted in TBS-MNGZ.

4.6 Sample preparation (II)

Purified protein complexes (streptavidin or TEV eluates) were reduced in 20 mM ammonium bicarbonate (Sigma) using 10 mM DTT (Sigma) and 2 mM TCEP (Life Technologies Europe BV) at 56°C, for 45 min. Afterwhich, they were alkylated with 55 mM iodoacetamide (Sigma) at room temperature, for 30 min. Reduced and alkylated protein complexes were further processed by overnight TCA / Acetone precipitation at -20 °C. Resulting pellets were dried by speedvac and solubilised in 0.05% RapiGest SF (186002122; Waters AB, Sweden) at 60 °C for 30-45 minutes. Sequencing Grade Modified Trypsin (0.25 - 0.5 µg, V5111, Promega AB) was utilised for overnight digestion of the samples at 37 °C.

4.7 Filter-aided sample preparation (FASP) (III)

The Filter-aided sample preparation protocol is modified from a previously described method (Wisniewski et al., 2009). Briefly, 10 kDa filters (Amicon Ultra 0.5) were washed twice with 300 µL of ultrapure Milli-Q water and centrifuged for 15 minutes at 18,000 rpm. Similar wash and centrifugation steps were performed three times with 0.1M NaOH and Milli-Q water. Before loading the sample, filters were washed once with 300 µL of urea buffer (UB) and centrifuged for 20 minutes. Reduced / alkylated crude lysates (10 µg) were loaded onto a filter with 300 µL of urea buffer (UB) and centrifuged as above. The filter was subjected to several washes (at least 5 times) with 300 µL of urea buffer (UB), followed by centrifugation. Overnight digestions were performed by directly applying Lys-C or Trypsin solutions to the filters, at room temperature, with shaking. Filters were first subjected to Lys-C digestion using 10 µL UB, 30 µL Milli-Q water and 20 µL Lys-C solution (0.05 µg / µL). The filter was centrifuged as above to collect Lys-C digested peptides (flow through) and store them in the cold room. The following day, filters were also digested with trypsin with 70 µL of 100 mM Ammonium bicarbonate, 50 µL Milli-Q water and 20 µL Trypsin solution (0.05 µg / µL Tryptic peptides (flow through) were collected by centrifugation, followed by further elution using 50 µL of 0.5M NaCl. Tryptic peptides from all elution steps were pooled and processed on Zip-Tip C18 reversed phase resin (Millipore, www.millipore.com/ziptip), for purposes of desalting, concentration and purification, as suggested by the manufacturers.

4.8 nano-LC / ESI / MS/MS analysis (II, III)

Tryptic peptides in water with 0.1% TFA were applied to an RP-18 precolumn (nanoACQUITY Symmetry® C18, 186003514; Waters AB) and separated on a nano-HPLC RP-18 column (nanoACQUITY BEH C18, 186003545; Waters AB) using an acetonitrile gradient (0 % - 60 % ACN in 120 min.), in the presence of 0.05% formic acid at a flow rate of 150 nl/min. The column outlet was directly coupled to the ion source of the spectrometer working in the regime of data dependent MS to MS/MS switch. A blank run ensuring lack of cross contamination from previous samples preceded each analysis.

4.9 Bioinformatic analyses (I, II, III)

The database for annotation, visualization and integrated discovery (DAVID; http://david.abcc.ncifcrf.gov/) (Huang da et al., 2009) and ClueGO (Bindea et al., 2009) were utilised to perform functional annotations analyses of NCL proteins. Putative palmitoylation sites on PPT1 IP were predicted using the *c*lustering and *s*coring *s*trategy algorithm (CSSPalm 4.0; http://csspalm.biocuckoo.org/index.php). Assignment of disease phenotypes associated with proteins of interest was based on information from the online mendelian inheritance in man (OMIM; http://www.omim.org/) database. We also utilised the mouse genome informatics database (MGI; http://www.informatics.jax.org/) database to search for unique mouse gene (GeneID) identifiers associated with *Ctsd*$^{-/-}$ differentially regulated proteins. In order to assign the mouse genes with their human orthologs, the NCBI homologene (http://www.ncbi.nlm.nih.gov/sites/homologene/) database was employed. Human orthologs of the mouse differentially expressed genes were further connected using the UniHI database (www.unihi.org) (Chaurasia et al., 2007) and GeneMANIA (www.genemania.org) (Mostafavi et al., 2008; Warde-Farley et al., 2010). Data from the Human Gene Atlas (http://biogps.gnf.org/) was utilised to analyse brain specific gene expression with analysis UniHi Express. Human orthologs of the mouse differentially expressed proteins were also linked to known pathways by the pathway interaction database (PID; http://pid.nci.nih.gov/PID/index.shtml). Mammalian protein complexes associated with the CLN3 / CLN5 interactomes were assessed using the comprehensive resource of mammalian protein complexes (CORUM; http://mips.gsf.de/genre/proj/corum/index.html) database. Global gene / protein networks associated with NCL proteins were functionally coupled by FunCoup database (http://FunCoup.sbc.su.se). CLN3 / CLN5 bridging proteins were connected and filtered for high expression in the brain, using the human integrated protein-protein interaction reference (HIPPIE) database (http://cbdm.mdc-berlin.de/tools/hippie/information.php).

5 Results and discussion

5.1 The Synaptic proteome of cathepsin D knockout mice (I)

The cathepsin D (*CTSD*) gene encodes a lysosomal aspartyl protease, that is mutated to cause congenital NCL in human (*CLN10*, OMIM: 610127) (Fritchie et al., 2009; Siintola et al., 2006). Moreover, CTSD is also involved in age-related neurodegenerative disorders, including: Alzheimer's (Nakanishi, 2003; Schuur et al., 2011), Parkinson's (Sevlever et al., 2008) and Creutzfeldt-Jakob diseases (Bishop et al., 2008). Based on previous experiments that showed deficits in synaptic trafficking or recycling in *Ctsd*$^{-/-}$ mice (Koch et al., 2011), we examined their synaptic proteome by quantitative mass spectrometry.

Our method of choice was isobaric tags for relative and absolute quantitation (iTRAQ) coupled with mass spectrometery, which is one of most powerful methodologies for simultaneous identification and quantitation of hundreds of proteins (Zieske, 2006). The iTRAQ reagent labels primary amines on peptides, hence theoretically facilitating the tagging of most tryptic peptides without loss of information from samples involving posttranslational modifications. Moreover, the multiplexing capacity of the technology affords increased throughput of the MS runs, thereby providing additional statistical validation within a given experiment.

5.1.1 Protein profiling of synaptosomal fractions from *Ctsd*$^{-/-}$ mouse brains

Synaptic alterations are a characteristic feature of most NCL mouse models, including the *Ctsd*$^{-/-}$ mice (Kielar et al., 2009; Kim et al., 2008; Virmani et al., 2005; Wishart et al., 2006). The synaptic pathology of *Ctsd*$^{-/-}$ mice has been demonstrated to particularly occur in the somatosensory cortex and thalamic ventral posterior medial / lateral nucleus (VPM/VPL); where the synaptic density is reduced during the late-symptomatic stages of disease (Partanen et al., 2008). Finally, loss of synapses is accompanied by aggregation of pre-synaptic proteins, including α-synuclein and SNARE proteins (Cullen et al., 2009; Partanen et al., 2008).

In an effort to probe the molecular mechanisms underlying cathepsin D deficiency, we isolated synaptosomal fractions from *Ctsd*$^{+/+}$ and *Ctsd*$^{-/-}$ mouse brains

at postnatal day 24 (P24) and analysed their protein profiles by iTRAQ. Over 600 proteins were identified in this study, including: synaptosomal proteins, as well as proteins involved in demyelination, accumulation of storage material and various signalling cascades in the brain. 43 of the identified proteins were differentially expressed between *Ctsd*$^{-/-}$ and wild type mice (Publication I: Supplementary Table 1). The differentially expressed proteins included some known brain specific markers, such as the myelin proteins, proteolipid 1 (Plp1/PLP1) and myelin basic protein (Mbp/MBP), which are less prominent in *Ctsd*$^{-/-}$ brains in comparison to the wild-type (Mutka et al., 2010).

A combination of protein profiling and brain gene expression data from iTRAQ experiments and the human gene atlas, respectively, was utilised to generate a dynamic map of cathepsin D related processes in the brain. Several functional modules associated with differentially expressed proteins in *Ctsd*$^{-/-}$ mouse brains, including: mitochondrial energy metabolism, vesicular transport, intergrin mediated signalling, myelin sheath, G-protein and microtubule assemblies (Publication I: Figure 3), were revealed in this work. Interestingly, 25% of the differentially expressed proteins were implicated in various brain disorders i.e. seizures and ataxia (Publication I: Figures 2B and 3); Alzheimer's disease and depression (Publication I: Figure 3); and schizophrenia (Martins-de-Souza et al., 2009). Quantitative proteomics profiling of brain regions has been successfully applied to studies of neurodegenerative disorders (Martin et al., 2008; Martins-de-Souza et al., 2009; Rhein et al., 2009).

5.1.2 iTRAQ data links CTSD deficiency to cytoskeletal alterations in the brain

The 43 differentially expressed proteins in *Ctsd*$^{-/-}$ mice were subjected to functional clustering analysis using the database for annotation, visualization and integrated discovery (DAVID) (Huang da et al., 2009). This analysis indicated associations to cell projection organization (GO:0030030), microtubule-based process (GO:0007017), and cytoskeleton organization (GO:0007010), within the most enriched cluster (Publication I: Figure 2A). Most differentially expressed proteins were significantly down-regulated in the *Ctsd*$^{-/-}$ mouse brain in comparison to wild type control (Publication I: Figure 2A). Data from iTRAQ experiments showed down-regulation of Microtubule associated protein 2 (MAP2), a protein involved in microtubule assembly and stability. Differential expression of the protein in *Ctsd*$^{-/-}$

mouse brains was validated by Western blot analysis and immunohistochemistry (Publication I: Figure 4B), in which diminished MAP2 staining in the hippocampus was demonstrated. Intriguingly, a similar decrease in MAP2 staining was observed in a mouse model of Alzheimer's disease (McKee et al., 1989).

5.1.3 Role of acetylation on microtubular interactions in neurons

α-tubulin acetylation was previously mapped to lysine 40 (LeDizet and Piperno, 1987) inside the microtubule polymer (Nogales et al., 1998), contrary to where most known interactions between microtubules and their associated proteins occur on the outer surface. Given the spatial separation of the modification and interaction sites, a potential role of acetylation in encoding microtubule functions seemed implausible. However, recent identification of several novel acetylation sites on tubulin (Choudhary et al., 2009) including some on the outer surface of the polymer, refocused attention on the role of acetylation in interactions between microtubules and microtubule associated proteins.

We observed up-regulation of acetylated TUBA1A in *Ctsd*-deficient mouse brains (Publication I: Figure 4A), from our iTRAQ experiments. Posttranslational modifications, including acetylation were suggested to mediate interactions between microtubules and their associated proteins (Fukushima et al., 2009). Modified microtubules are postulated to have roles in maintenance of neuronal morphology, as well as neurite outgrowth and maturation. Additionally, acetylation of alpha-tubulin at lysine 40 is critical for radial migration and branching of cortical projection neurons (Creppe et al., 2009). Histone acetyltransferase composed of ADP-ribosylation factor domain protein 1 (ARD1) in complex with N-terminal acetyltransferase 1 (NAT1) (Park and Szostak, 1992) could acetylate α-tubulin, and was determined to be critical for dendrite extensions and arborization (Creppe et al., 2009). Acetylation of microtubules or other substrates of ARD1-NAT1 is important for the early stages of neuronal development and maybe an essential signal for neuronal migration and differentiation, as well as synaptic targeting. Alterations in microtubular interactions were also documented in Parkinson's and Huntington's diseases (Dompierre et al., 2007; Outeiro et al., 2007; Suzuki and Koike, 2007).

5.1.4 Aberrant Focal adhesion sites and cell migration deficits in *Ctsd*$^{-/-}$ cells

Deficits in spatial orientation and alterations in cell migration of *Ctsd*$^{-/-}$ cells may be directly attributed to aberrant cell adhesion points. In order to clarify this possibility, we probed the cellular localization of focal adhesion kinase (FAK), a key protein of the focal adhesion sites. Focal contacts provide a structural link between the actin cytoskeleton, extracellular matrix and signalling proteins during cell adhesion, spreading and migration (Bershadsky et al., 2003; Ridley et al., 2003). FAK exhibits both scaffolding and signalling functions. Autophosphorylation of Tyrosine 397 provides a docking site for SH2-containing proteins, whereas regulation of its catalytic activity requires activation of Tyrosines 576 and 577 (Calalb et al., 1995; Schaller, 2001).

Based on immunofluorescence analyses of *Ctsd*-deficient mouse and human fibroblasts (Publication I: Figures 7 and 8), FAK, vinculin, phosphorylated Tyr and β1-integrin, α5-integrin, talin, respectively, were weakly immunostained in comparison to the wild type cells. These findings indicated defects in formation of focal adhesion sites in *Ctsd*-deficient cells that probably affect their movement. FAK is involved in regulation of axonal branching and synapse formation (Rico et al., 2004), shaping migrating growth cones and assembly of axo-dendritic contacts (Stagi et al., 2010). Failure to form focal adhesion sites is therefore a potential hindrance to neuronal development and synaptogenesis in mature neurons.

Bioinformatic analyses revealed integrin signalling as one of the pathways affected in *Ctsd*$^{-/-}$ mouse brains (Publication I: Figure 3). Activation of FAK is known to regulate integrin signalling, hence our motivation to stain human fibroblasts for β1-integrin (ITGB1), α5-integrin (ITGA5), and talin (TLN), an adaptor protein in the integrin complex (Publication I: Figure 8). Human fibroblasts were grown on fibronectin in order to induce integrin-clustering and focal adhesion site formation. Previous experiments with mice fibroblasts were validated by findings in control human fibroblasts, which also showed integrin clustering and presence of focal adhesion sites at the plasma membrane. Immunostaining of β1-integrin was however globally distributed within the *Ctsd*-deficient human fibroblasts. Staining for α5-integrin and talin was weak throughout the cells. Focal adhesion sites therefore appear aberrantly localised in the *Ctsd*-deficient cells.

5.2 Mapping the CLN3-CLN5 Interactome in the brain (II)

Mutations in *Cln3* and *Cln5* cause CLN3 disease / Juvenile NCL (JNCL, OMIM: 204200) (Consortium, 1995) and CLN5 disease / Finnish variant LINCL (vLINCL$_{Fin,}$ OMIM: 256731), respectively. CLN3 disease is the most common childhood neurodegenerative disorder (Consortium, 1995), whereas CLN5 disease is especially enriched in the Finnish population (Savukoski et al., 1998). NCL share similar pathological and clinical phenotypes, which suggests that they are probably connected at a molecular level. Previous work also supports this hypothesis (Vesa et al., 2002; von Schantz et al., 2008). Immunofluorescence microscopy, co-immunoprecipitation and *in vitro* binding assays in transiently transfected COS-1 cells, showed CLN5 interactions with CLN2, CLN3 (Vesa et al., 2002). Moreover, quantitative gene expression profiles and immunofluorescence stainings of 1 / 4 month old *cln1*$^{-/-}$ / *cln5*$^{-/-}$ mice cortex, showed aberrations in neuronal growth cone assembly (von Schantz et al., 2008). We therefore examined the CLN3 and CLN5 interactomes in SH-SY5Y human neuroblastoma cells by TAP-MS, in an effort to determine the extent of cross-talk among the two NCL proteins.

In order to map the CLN3-CLN5 interactome in the brain, we initiated a proteomic analysis of the tandem affinity purification (TAP)-tagged disease proteins and their associated complexes. The unbiased five step approach involved: construction of mammalian retroviral based expression vectors, generation of stably expressing cell lines, Tandem Affinity Purification (TAP), shot-gun sequencing of isolated protein complexes and functional annotation / interactome analysis (Publication II: Figure 1). Cellular models are particularly beneficial for TAP experiments because they are relatively inexpensive, faster to culture and easier to manipulate, in comparison to animal models (e.g. mice). However, isolation of protein complexes from the latter (TAP-tagged mice), for instance, would ensure that the bait and associated proteins are maintained at physiological levels and within their right stoichiometric amounts.

5.2.1 Identification of novel CLN3 and CLN5 IP

Proteins typically function as constituents of molecular complexes that perform various tasks within cells (Alberts, 1998). Consequently, understanding unknown functions of proteins requires examination of their complexes under native conditions. In this study, we utilised tandem affinity purification coupled to mass spectrometry (TAP-MS), for purposes of isolating and identifying CLN3 / CLN5 protein complexes along with their associated interacting partners. Tandem Affinity Purification is a dual-step method for purifying protein complexes from cell lysates or animal tissues that express TAP-tagged fusion proteins of interest (Puig et al., 2001; Rigaut et al., 1999).

We generated SH-SY5Y cells stably expressing CLN3 and CLN5 baits using N-terminal or C-terminal TAP-tagged vectors respectively, for use in TAP-MS experiments. Following TAP-MS, bioinformatics analyses with Significance Analysis of Interactome (SAINT) yielded 58 and 31 CLN3 or CLN5 IP, respectively (Publication II: Tables 1 and 2). As a proof of principle, 37% of CLN3 high confidence IP previously determined by another study were also recovered in our study (Behrends et al., 2010). Moreover, a subset of CLN3 IP (DBH, DPM1, SEC61A1 and SLC25A10) was validated in co-immunoprecipitation assays (Publication II: Figure 3) and dual Immunofluorescence confocal microscopy (Publication II: Supplementary Figure 2).

CDP-diacylglycerol synthase (CDS2), an enzyme that catalyses the conversion of phosphatidic acid to CDP-diacylglycerol and subsequently regulates available phosphatidylinositol for signalling, was one of the novel CLN3 IP that we identified. The enzyme is implicated in downstream G-protein signalling events because it provides the precursor for synthesis of phosphatidylglycerol, cardiolipin (inner mitochondrial membrane) and phosphatidylinositol (ER) (Inglis-Broadgate et al., 2005). Interestingly, treatment of autophagy defective *Cln3*-deficient mouse cerebellar cells with lithium (inositol monophosphatase inhibitor), partially recovered autophagy in these cells and diminished their vulnerability to cell death (Cao et al., 2006; Chang et al., 2011). Studies in myo-inositol monophosphatase (IMPase) mutants of *C. elegans* indicated that a reduction in phosphatidylinositol 4, 5-bisphosphate (PIP2) caused abnormal synaptic polarity (Kimata et al., 2012), thereby mechanistically linking phosphatidyl signalling to defects in autophagy, synaptic polarity and apoptosis.

5.2.2 Neurodegenerative disease phenotype of the CLN3-CLN5 Interactome

Mental retardation (MR) and epilepsy often occur concurrently because they have similar etiology. MR affects 1–3% of the human population and is characterized by deficits in intellectual function and adaptive behavior with onset in childhood. Family studies have highlighted the relatively large number of X-linked mental retardation (XLMR) cases, which probably explains the higher MR incidence in males (Tarpey et al., 2009). Several of the identified XLMR genes are involved in synaptic function (Humeau et al., 2009). Epilepsy is one of the most common neurological disorders, characterized by abnormal electrical activity in the central nervous system (CNS). It affects nearly 3% of the world's population, and its pathogenesis is attributed to several factors, including: trauma, virus infection, altered metabolic states, or inborn brain malformations (Tian and Macdonald, 2012). Most common genetic epilepsies, such as juvenile myoclonic epilepsy (JME) or childhood and juvenile absence are complex diseases (Michelucci et al., 2012; Steinlein, 2004).

Analysis of the CLN3-CLN5 interactome against the OMIM database, yielded 8 disease associated IP (Publication II: Supplementary Table 2), 6 of which were categorised with a neurodegenerative phenotype. The neurodegenerative disease associated proteins included those involved in mental retardation or epileptic seizures (CLN3, CLN5, STRA6, TECR, PHGDH and SLC25A22), schizophrenia (DBH) and dystonia parkinsonism (ATP1A3) (Publication II: Figure 5A). Mental retardation and epileptic seizures are both known clinical symptoms of NCL patients. Furthermore, a link to Huntington's disease and Parkinson's disease pathways was established from 4 CLN3 IP (SLC25A6, SLC25A5, SLC25A4 and UQCRC2) (Publication II: Figure 4C). Synuclein (SNCA) was shown to directly bind to CLN3 (Koenn, 2012) and also interacts with several CLN3 / CLN5 IP (Publication II: Supplementary Figure 5).

5.2.3 Molecular link between NCL proteins

Bioinformatic analyses and literature searches revealed potential interactions between several NCL proteins, reviewed by (Getty and Pearce, 2011; Kollmann et al., 2013). In accordance with other multifactor gene disorders, NCL proteins may be involved in cross-talk between many cellular pathways and result in similar mechanisms of neurotoxicity. Our recent studies on CLN3-CLN5 and CTSD interactomes (Koch et al., 2013; Scifo et al., 2013) pinpointed their inter-connections with proteins involved

in neurodegeneration, mental retardation and epileptic seizures, as well functional modules, which could be targeted pharmaceutically.

Given the novelty of most identified CLN3 and CLN5 IP, we employed FunCoup database in an effort to uncover their functional associations by network approaches. The statistical platform permits data integration from various model organisms, for purposes of establishing functional coupling between proteins in medium or high-throughput data sets (Alexeyenko et al., 2012). A total of 72 input genes were present in FunCoup, from which 67 were utilised to identify 649 high confidence functionally coupled pairs. For instance, 22% of CLN3 IP, including two novel ones (SCAMP3 and TECR) were predicted with high confidence to be functionally coupled with CLN3. Moreover, CLN5 was also predicted with high confidence to be functionally coupled to CLN3 and SEL1L. A high number of links amongst the functionally coupled proteins suggests a dense network of associations within the input genes and further implicates them in similar biological processes.

We finally subjected the CLN3-CLN5 bridging proteins (Publication I: Figure 5) to functional clustering by querying HIPPIE for their direct IP, followed by filtration for high expression in the brain (Schaefer et al., 2012). Most of the mitochondrial carriers were associated with proteins involved in autophagy, a pathway that regulates mitochondrial turnover and is known to be disrupted in CLN3 disease. Disruption of GABAergic neuronal populations in hippocampal CA2-CA4 regions of CLN3 and CLN5 patients, suggests a potential role of the autophagic pathway including GABA-receptor associated proteins in enhancing the neuropathology (Tyynela et al., 2004).

5.3 The human CLN1 (PPT1) Interactome in the brain (III)

The *CLN1* gene encodes CLN1 or Palmitoyl Protein Thiosterase 1 (PPT1), a soluble protein that when mutated is known to cause Infantile NCL (INCL, MIM#256730) or CLN1 disease (Hofmann et al., 2001). 45 different mutations are implicated in CLN1 disease (http://www.ucl.ac.uk/ncl/), including [R122W] and [T75P] that account for 98% of INCL cases in Finland and 13% of disease alleles in the USA, respectively (Das et al., 1998; Vesa et al., 1995). Based on literature review, 12 PPT1 IP have been determined by various experimental methods, i.e affinity capture-MS (Danielsen et al., 2011; Emanuele et al., 2011; Kim et al., 2011; Koch et al., 2007; Liu et al., 2012; Wagner et al., 2011), co-fractionation (Havugimana et al., 2012) and yeast two-hybrid assays (Wang et al., 2011). However, these studies utilised non-neuronal cells

to identify the PPT1 IP and their findings probably do not represent *in vivo* substrates of the enzyme in the brain. We therefore aimed to mimic the physiological neuronal cellular environment of PPT1 in the brain by isolating protein complexes from human SH-SY5Y neuroblastoma cells at near to endogenous levels.

5.3.1 Neurodegenerative disease causative proteins interact with PPT1

Proteomic analysis of PPT1 IP by single step affinity purification coupled to mass spectrometry (AP-MS) yielded nine neurodegenerative disease causative proteins (VCP, VAPB, CRMP1, DBH, VDAC2, CTSD, PDHA1, PDHB and DLAT) (Publication III: Figure 5). Two of the PPT1 IP in this category (VCP and DBH) were validated by co-imunoprecipitation (Publication III: Figure 3). Valosin-containing protein (VCP or p97) is a 97 kDa member of the type II AAA (*A*TPases *a*ssociated with a variety of *a*ctivities) ATPases, which are distinguished by their two conserved ATPase (AAA) domains (Neuwald et al., 1999). VCP is highly conserved across various species (e.g. known as VAT in archaebacteria, CDC48 in yeast, TER94 in *Drosophila*, p97 in *Xenopus*, and VCP in plants and mammals) (Frohlich et al., 1991; Koller and Brownstein, 1987; Pamnani et al., 1997; Peters et al., 1990). It is ubiquitously expressed in cells and has been suggested to function in various cellular processes, including: ubiquitin-dependent protein degradation, membrane fusion, ER-associated degradation, transcription activation, cell cycle control, apoptosis and molecular chaperone (Wang et al., 2004). Mutations in the valosin-containing protein (VCP) cause an autosomal dominant inclusion body myopathy associated with Paget's disease of the bone and frontotemporal dementia (OMIM: 605382) (Schroder et al., 2005; Watts et al., 2004). VCP is particularly of great interest to researchers across various disciplines because of its unusually diverse functions.

Human dopamine β-hydroxylase (DBH) is a 617 amino acid (78 kDa) single-pass type II transmembrane glycoprotein (SwissProt # P09172) related to the copper type II, ascorbate-dependent monooxygenase family. The protein comprises of N-terminal cytoplasmic (aa 1-16), transmembrane (aa 17-37) and luminal (aa 38-617) regions. A DOMON (dopamine β-monooxygenase N-terminal) domain (aa 51-169) and two consecutive monooxygenase motifs (aa 214-523) are part of the luminal region. Unlike other members of the family (e.g tyrosine hydroxylase, TH and phenylethanolamine N-methyltransferase, PNMT) which are cytosolic, DBH is localised in membranes of secretory vesicles (Chen et al., 2003; Gearhart et al.,

2002). It is expressed in noradrenergic nerve terminals and adrenal medullary chromaffin cells, and serves as a catalyst for the conversion of dopamine to norepinephrine (Man in 't Veld et al., 1987; Robertson et al., 1986) . Dopamine beta-hydroxylase deficiency (OMIM: 223360) is caused by a mutation in the *DBH* gene encoding DBH (Kim et al., 2002).

5.3.2 PPT1 interacts with PDH_C and ATP synthase complexes

Amongst the novel PPT1 IP identified in this study was PDHA1, PDHB, DLAT and DLD (Pyruvate dehydrogenase complex) and ATP5B, ATP5A1 (ATP synthase complex) (Publication III: Figure 5, Table 1 and Supplementary Table 1). The pyruvate dehydrogenase complex (PDH_C) catalyzes irreversible oxidative decarboxylation of pyruvate to acetyl CoA, thereby linking glycolysis to the citric acid cycle. Multiple copies of 3 separate enzymes: pyruvate dehydrogenase (PDH, E1: 20-30 copies), dihydrolipoamide S-acetyltransferase (DLAT, E2: 60 copies) and dihydrolipoamide dehydrogenase (DLD, E3: 6 copies), constitute the pyruvate dehydrogenase complex. The complex also requires 5 different coenzymes: CoA, NAD^+, FAD^+, lipoic acid and thiamine pyrophosphate. A deficiency of PDHc deficiency is suggested result in lactic acidaemia and insufficient energy production, probably due to the presence of residual pyruvate and lactate that are insufficiently removed from cells. The neurological disease spectrum associated with PDHc ranges from fatal lactic acidosis in newborns, intermittent ataxia in milder forms, to chronic neurological dysfunction with mental retardation (Imbard et al., 2011).

5.3.3 Putative roles of PPT1 in neuronal migration and axonal guidance

PPT1 also interacts with microtubule associated protein 1B (MAP1B) (Publication III: Figure 5, Table 1 and Supplementary Table 1), a protein that is highly expressed in axons of developing neurons (Black et al., 1994) and is suggested to regulate neuronal migration and axonal guidance (Gonzalez-Billault et al., 2004). A role of MAP1B in axonal guidance was showed by histological examination of homozygous *Map1b*-deficient (*Map1b*$^{\Delta 93}$) mice brains (Meixner et al., 2000). Most of the *Map1b* gene was removed in these deletion mutants that could not express Map1b from either regular or alternative transcripts. Expression of Map1b: heavy / light chains and N-terminal fragments was determined to be completely abolished in these mutants.

Histological staining of brain tissues from these mice revealed agenesis of the corpus callosum and formation of Probst bundles (Meixner et al., 2000). These features were present in all homozygous *Map1b*-deficient mice brains, but absent in either heterozygotes or wild-type controls thus suggesting an important role of MAP1B in corpus callosum genesis.

During development, expression of MAP1B occurs in the axons, dendrites, and growth cones of the CNS (Black et al., 1994). However, the observed defect in axonal guidance is restricted to the corpus callosum and the hippocampal commissure. It is probable that a single mechanism involving MAP1B is indespensable for axonal guidance in the corpus callosum, whereas other back-up mechanisms maybe employed for the guidance of most axons throughout the CNS. MAP1B may participate in signal transduction aided by laminin expressed on the cell surface of midline glia cells (Liesi and Silver, 1988). A role of MAP1B in laminin-enhanced axonal growth has been demonstrated *in vitro* assays (DiTella et al., 1996). Moreover, mice deficient in p35 (suggested to participate in regulation of laminin-induced MAP1B phosphorylation ((Paglini et al., 1998)), also exhibit axonal guidance defects in the corpus callosum (Kwon et al., 1999). MAP1B may regulate corpus callosum formation via the laminin / integrin-mediated cell adhesion signalling pathways.

The interaction of PPT1 with MAP1B probably facilitates depalmitoylation of the latter thereby releasing the protein to participate in laminin induced axonal guidance critical for corpus callosum genesis.

5.3.4 PPT1 is linked to the dopamine receptor mediated signalling pathway

Dopamine β-hydroxylase (DBH) is a PPT1 IP (Publication III: Figure 5, Table 1 and Supplementary Table 1) involved in the dopamine receptor mediated signalling pathway. The enzyme is an oxidoreductase that catalyses the conversion of dopamine to norepinephrine and therefore regulates norephinephrine / dopamine ratio in noradrenergic neurons. Regulation of firing patterns and subsequent dopamine release are directed by noradrenergic neurons (in the locus coeruleus), which project to dopamine neurons in the ventral tegmental area (VTA) (Grenhoff et al., 1993; Jones and Moore, 1977; Jones and Yang, 1985; Phillipson, 1979). Based on radioligand binding assays, *Dbh*-deficient mice were shown to have an increased density of striatal high affinity state (D_1 and D_2) dopamine receptors and were hypersensitive to

amphetamine or cocaine induced locomotion (Schank et al., 2006; Weinshenker et al., 2002).

Nigrostriatal dopaminergic projections originate from dopamine-synthesising A9 neurons of the midbrain substantia nigra (pars compacta) and innervate the dorsal striatum (caudate-putamen). They constitute one of the four major dopaminergic pathways and are an important regulator of motor control circuitry (Smith and Villalba, 2008). Progressive loss of the A9 neurons in Parkinson's disease (PD) patients, accounts for their characteristic symptoms of bradykinesia and rigidity. Depletion of dopamine results in degeneration of the nigrostriatal pathway which is apparent in PD patients.

Dopaminergic synaptic transmission in the brain involves binding to five known dopamine receptor subtypes ($D_{1\text{-}5}$) (Dearry et al., 1990; Sokoloff et al., 1990; Sunahara et al., 1991; Van Tol et al., 1991; Zhou et al., 1990). Dopamine receptors are classified according to their molecular structure and pharmacological properties (Jackson and Westlind-Danielsson, 1994). D_1-like dopamine receptors (D_1 and $D_{5)}$ activate adenylyl cyclase and cyclic adenosine monophosphate (cAMP) production via Gs/olf proteins (Dearry et al., 1990; Sunahara et al., 1991; Tiberi et al., 1991; Zhou et al., 1990) when stimulated, whereas the D_2-like receptors ($D_{2,}$ D_3 and D_4) inhibit adenylyl cyclase via Gi/o proteins (McAllister et al., 1995; Onali et al., 1985; Potenza et al., 1994; Tang et al., 1994) and activate phospholipase Cβ (PLCβ) (Hernandez-Lopez et al., 2000). Both D_1 and D_2 -like dopamine receptors regulate calcium signalling (Missale et al., 1989) and stimulate mitogen-activated protein kinase pathways (Cai et al., 2000; Zhen et al., 1998).

Interestingly, DBH was also identified as common interactor of both CLN3 and CLN5 (Publication II: Figure 5, Tables 1 and 2). It is plausible that the common phenotype amongst NCL disease genes is partly modulated by involvement in the dopamine receptor mediated signalling.

6 Conclusions and future prospects

The first goal of this thesis work was to systematically characterize the *Ctsd*$^{-/-}$ mice synaptic proteome, in an effort to unravel mechanisms underlying cathepsin D deficiency. Using a quantitative proteomics approach, we observed that several functional modules, such as: mitochondrial energy metabolism, vesicular transport, intergrin mediated signalling, myelin sheath, G-protein and microtubule assemblies were linked to cathepsin D deficiency in these knockout mice. We specifically observed alterations in microtubule associated cytoskeleton and cell projection organization which might constitute a preliminary phase in the synaptic aberrations and neurodegeneration characterized by cathepsin D deficiency. It would be worthwhile to intracranially inject *Ctsd*$^{-/-}$ mice with adeno-associated viral vectors expressing MAP2, ITGB1 and ITGA5, to determine if they can partially rescue the microtubule and integrin associated altered cytoskeleton phenotype observed in these mice.

We further applied tandem affinity purification- MS techonology towards the isolation and identification of CLN3 and CLN5 protein complexes in human neuroblastoma cells. We subjected our dataset to stringent Significance Analysis of Interactome in order to minimise false positives associated with various high throughput strategies. Functional annotation of the combined CLN3-CLN5 interactome showed enrichment in proteins associated with neurodegenerative diseases, vesicular trafficking and the lipofuscin interactome. Besides confirming known roles of CLN3 in transmembrane transport, lipid homeostasis and neuronal excitability, this work also identified putative involvement of the protein in G-protein signalling, as well as protein folding / sorting in the ER. Moreover, we also identified several proteins (mostly mitochondrial solute carriers) that interact with both CLN3 and CLN5, which is suggestive of common underlying molecular mechanisms associated with disease pathogenesis in the two NCL proteins.

Another aim of this work was to characterise the *in vivo* substrates of human PPT1 in the brain by a single step affinity purification coupled to mass spectrometry (AP-MS) approach. PPT1 protein complexes were processed by filter assisted sample preparation (FASP) and analysed on two MS instruments. This work yielded several PPT1 IP including: neurodegenerative disease causative proteins, as well as pyruvate dehydrogenase and mitochondrial ATP synthase complexes. A subset of the PPT1 IP was validated in co-immunoprecipitation assays. However, the proteomic data not only confirms previously suggested roles of PPT1 in axon guidance and lipid metabolism, but also implicates the enzyme in putative new roles, such as:

involvement in neuronal migration and dopamine receptor mediated signalling pathway.

Synaptic alterations are well documented in most NCL mouse models and so the finding that DBH (enzyme implicated in dopamine receptor mediated signalling) is a common interacting partner to PPT1, CLN3 and CLN5, may be of therapeutic importance. As such, future experiments might involve overexpression of DBH in *Cln1*$^{-/-}$, *Cln3*$^{-/-}$ and *Cln5*$^{-/-}$ mice brains, for purposes of modulating dopaminergic synaptic transmission.

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