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The contribution of multicellular model organisms to Neuronal Ceroid Lipofuscinosis research

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Highlights

- Model organisms highlight mechanisms, protein functions and relevant pathways
- A range of model organisms with various attributes enable *in vivo* experimentation
- Therapeutic testing for NCLs relies on a range of suitable disease models

Abstract

The NCLs (neuronal ceroid lipofuscinosis) are forms of neurodegenerative disease that affect people of all ages and ethnicities but are most prevalent in children. Commonly known as Batten disease, this debilitating neurological disorder is comprised of 13 different subtypes that are categorized based on the particular gene that is mutated (*CLN1-8, CLN10-14*). The pathological mechanisms underlying the NCLs are not well understood due to our poor understanding of the functions of NCL proteins. Only one specific treatment (enzyme replacement therapy) is approved, which is for the treating the brain in CLN2 disease. Hence there remains a desperate need for further research into disease-modifying treatments. In this review, we present and evaluate the genes, proteins and studies performed in the social amoeba, nematode, fruit fly, zebrafish, mouse and large animals pertinent to NCL. In

particular, we highlight the use of multicellular model organisms to study NCL protein function, pathology and pathomechanisms. Their use in testing novel therapeutic approaches is also presented. With this information, we highlight how future research in these systems may be able to provide new insight into NCL proteinF functions in human cells and aid in the development of new therapies.

Keywords

Batten disease; Neuronal Ceroid Lipofuscinosis; lysosomal storage disorder; model organism; disease mechanism; pathology; experimental therapy;

Abbreviations

ALS, amyotrophic lateral sclerosis; CNS, central nervous system; CT, computed tomography; CV, contractile vacuole; dpf, days post-fertilisation; ECG, electrocardiography; EEG, electroencephalography; EMA, European Medicines Agency; EOG, electrooculography; ER, endoplasmic reticulum; ERG, electroretinogram; ERT, enzyme replacement therapy; FTLD, frontotemporal lobar degeneration; hpf, hours post-fertilisation; ICV, intracerebroventricular; KRS, Kufor-Rakeb syndrome; MO, morpholino oligonucleotide; MRI, magnetic resonance imaging; NCL, Neuronal Ceroid Lipofuscinosis; NHP, non-human primates; REMI, restriction enzyme-mediated integration; RNAi, RNA interference; UPS-ubiquitin proteasome system; US FDA, United States Food and Drug Administration; WGS, whole genome sequencing.

1. Introduction

Neuronal Ceroid Lipofuscinosis is a group of fatal neurodegenerative diseases that are characterised by lysosomal storage of ceroid lipofuscin. Each disease is designated CLN followed by a number that refers to a gene. Recessive mutation in one of those genes causes a specific type of NCL, for example mutations in the *CLN3* gene cause CLN3 disease. Each form of NCL has a characteristic age of onset and speed of progression, as well as specific storage material and appearance under electron microscopy. Although the central nervous system is predominantly affected, pathology is seen in peripheral tissues as well[1, 2]. Currently, one approved treatment exists for CLN2 disease – an enzyme replacement therapy infused directly into the brain [3]. A few gene therapies and small molecule treatments are in clinical trials. These are reviewed elsewhere [4, 5]. Given that the NCLs are likely to end up affecting other parts of the body even if the brain or retina is successfully treated, there is still a huge need develop further treatments for this debilitating and devastating disease. Animals are required for drug testing and large animal disease models are particularly useful for examining penetration of treatments as their brains are nearly human size. Multicellular and animal models can also shed a light on pathological processes and the function of CLN proteins for which the function is unknown, with such information leading to potential drug target identification.

The use of multicellular models and live animals in NCL research is crucial. Using live animals enables researchers to investigate the effects of single gene loss on whole tissues and organisms. Multi-cellular organisms are also needed for investigating pathology, treatment efficacy, tissue-specific toxicity and immunological reactions, as

well as determining the relevance of the blood-brain barrier, and how a treatment is metabolised. If experimental results are the same across several model organisms, this indicates that the researchers have identified a conserved feature (rather than a species-specific feature) and significantly raises confidence in the relevance of that finding.

The experimental use of animals protected by legislation must be sufficiently justified by results from experiments using cells or animals that are not protected by legislation. These include invertebrates and embryonic vertebrates, and this review highlights these ethical issues and demonstrates the relative uses of each organism at relevant stages of development.

Traditional drug discovery methods start either by identifying a target in cellular models and then identifying compounds that modulate that target (target-based screening), or by identifying compounds that ameliorate a phenotype (phenotypic screening) [6]. In both cases, many thousands of compounds may be screened. Target-based screening requires the knowledge of a target thought to be centrally involved in the disease, but, due to a lack of understanding of nearly all forms of NCL, targets have not been identified. Indeed, although the function of some NCL proteins are known (e.g. lysosomal enzymes such as Protein palmitoyl thioesterase 1 (PPT1), Tri-peptidyl peptidase 1 (TPP1) and Cathepsin D (CTSD)), the function of many other NCL proteins remains elusive, hampering efforts to identify targets. To perform phenotypic screening, an assay is used that is thought to be relevant to the disease or the function of the protein, but this can be hard to judge when little is known about the disease and the screening is limited by being carried out in cellular models. For NCL, phenotypic screening has included assays for apoptosis, for example [7]. Once a set of compounds has been identified, animals are then used to verify which compounds improve the disease in the animal and, unfortunately, many compounds fail at this point. Of those compounds that do show improvement in the animal model, several will fail at clinical trial because the result (efficacy or toxicity) in the animal was not representative of humans [8]. It has been suggested that employing animal models such as the social amoeba, nematodes, fruit flies, or zebrafish earlier in the phenotypic screening process, the use of multiple animal models, and the use of phenotypes that are representative of clinical signs in patients will contribute to reduce the numbers of failures at clinical trial [9, 10]. However, it is unlikely that the numbers of compounds that can be screened using animal models would ever be as great as when using cells. A further issue is that it is difficult to identify the target of the lead compound. This is often required before approval of a new drug is granted but can be waived for rare diseases where a treatment is not yet available.

Another route to treatment is the replacement of a protein known to be missing, for example by gene therapy or enzyme replacement therapy. This could be considered a target-based approach. In this case, cellular models, invertebrates and non-mammalian vertebrates are usually bypassed and the therapy is tested first in a mammalian model, usually starting with a mouse model as they are the cheapest and easiest to work with. However, the physiology and pharmacodynamics of larger animals are more likely to approximate the human condition. Therefore, it is usual to also test these therapies on larger animals such as dogs or sheep before clinical trials in patients.

In this review, we first provide a brief discussion on each of the model systems that will be presented in the review and why they are useful for NCL research (Section 2). In the sections that follow (Sections 3-15), we discuss each of the NCL subtypes in turn. For each subtype, we present details on the model systems that have been used to study that particular subtype of the disease, and highlight the recent advances made, both fundamental and applied, using those systems. In the final section (Section 16), we provide concluding remarks on the future of NCL research using model systems.

2. Animal models: characteristics and ethical considerations

2.1 Large animals and veterinary species

Large animal models of NCL are a critical in understanding disease pathogenesis and testing disease modifying agents. NCLs have been identified in dogs, cats, sheep, cattle, horses and most recently a monkey, however many of these are isolated apparently single cases reported in veterinary journals and not always with confirmed genetic diagnosis. Of those that have been established as research colonies, the dog and sheep models are the best studied and will form the major focus for large animal discussion in this review, especially work described since the last Biochimica et Biophysica Acta - Molecular Basis of Disease review [11].

In terms of understanding biology, pathogenesis and developing treatment strategies, each model system has key advantages and disadvantages. The large animal models replicate more of the clinical features of the human conditions and have gyrencephalic brains (highly folded cortices with gyri and sulci) more similar to humans. However, these models are generally more expensive to keep, restricted to certain facilities with housing capacity and limited to seasonality for collection of tissues/breeding. The longer lifespan and development of disease in many models also makes these more challenging models, though together with small animals and cellular systems provide a key component in the NCL research network.

Historically, sporadic cases of NCL in large animals was identified by veterinary pathologists [12, 13]. Characteristic clinical signs of NCL were confirmed by biopsy/pathology for autofluorescent storage material and by electron microscopy to classify membrane structure for a preliminary definition of probable subtype. Since the elucidation of NCL genes, most large animal models can be identified by genotype. This diagnosis is still problematic in some species with incomplete genome coverage, however in dogs, exome and / or whole genome sequencing (WGS) analysis allows most forms to be distinguished. The genetically characterised forms of NCL in large animal models are shown in Table 1. Those that have been recently identified and / or established as research colonies are discussed further in relevant gene-associated subsections.

Genetic modification technologies have been instrumental for the development of animal models, especially in mice. The technology has been slower to develop in large animals, though is possible [14, 15]. Several new large animal models, including pig models of CLN2 (J. Weimer, Sanford Research, SD, personal communication) and CLN3 disease [16] and a sheep model of CLN1 disease [17], are currently being developed. These advances will, importantly, allow the generation of animal models expressing allele -specific mutations identified in patient populations, especially for those forms that differ dramatically in phenotype depending on underlying genotype.

Pre-clinical testing in large animal models is often required prior to clinical trial approval, at least to show safety and tolerability of any gene/drug therapy. At minimum these therapies require testing in non-human primates (NHP), most often in "wildtype' species as, other than for CLN7 [18] no NHP models of other NCLs currently exist. These will be discussed in more detail where they have been used in specific preclinical studies.

2.2 Rodents

Rodents are probably the most commonly used animal model for studying human neurological diseases, among which mouse models are the most popular and economical option. The mouse and human genomes share around 85% identity, and a large number of proteins are evolutionally conserved between the mouse and human both in structures and functions. For monogenetic diseases, genetically modified mouse models provide a powerful tool for both studying human disease pathogenesis and testing potential therapeutic strategies. In particular, as mice have been employed in neuroscience research for a long time, a variety of mouse-based research tools, methods and databases have been well established and widely employed for neurobiological and behavioural studies.

Almost all types of NCL have available mouse models with corresponding gene alterations, except for CLN14 disease (Table 2). Most of these models are genetically modified mouse lines; except for two naturally occurring mouse NCLs (*mnd* and *ncl*f), both corresponding to human NCL subtypes [19-21] [22-25]. These mouse models recapitulate many typical NCL features (motor and/or visual impairments, neuronal lipofuscin accumulation confirmed by light and electron microscopic analysis, gliosis, neuro- and/or retinal degeneration, *etc*.) and utilising these models has markedly advanced our understanding of NCL pathogenesis and also promoted delineating the functions of the proteins mutated in NCLs. Moreover, mouse models are particularly useful for investigating potential therapeutic approaches. The small body size/weight, ease of handling and low cost makes the mouse an ideal mammalian model for testing novel therapies or drugs with a decent sample size but not over-expenditure. However, due to dramatic differences in the lifespan, brain/body size and structures between the mouse and human, pre-clinical therapy testing with large animal models is still irreplaceable before proceeding to clinical trial, especially for testing long-term efficacy, safety and tolerability. Available murine models of all NCLs as well as corresponding use in pre-clinical studies are listed in Table 2. NCL research advances using mouse models will be discussed in detail in the following sections of this article.

2.3 Zebrafish

In the 1980s, George Streisinger (Univ. Oregon, USA) was the first researcher to substantially promote the use of zebrafish, *Danio rerio,* for genetics and developmental biology. Since then, publications have increased from a handful each year to about 5000 in 2016 (reviewed in [26]). The main driver for the uptake of this model organism into laboratories is that is the only vertebrate that is transparent during embryonic and larval stages, and can be maintained as transparent using either chemical or genetic tricks. Add in features such as external fertilisation and development, easy care, easy generation of large numbers, breeding all year round,

ability to breed for at least a year, and a diploid genome, and you have a powerful vertebrate model organism for developmental genetics. Furthermore, zebrafish embryos and larvae prior to free-feeding at 5 days post-fertilisation (dpf) are not protected species in relation to animal experimentation. As zebrafish hatch from their chorion at 3dpf, are swimming and have developed several behaviours, they are useful for motor function and behavioural studies from this age. Many zebrafish mutants have an earlier onset phenotype than their mammalian equivalents (e.g. zebrafish versus mouse and canine models of CLN2 disease [27-29]), enabling quicker results.

Although genetic manipulation is not as simple as for invertebrate model organisms, many of the methods generated in *Drosophila* have been translated to the zebrafish. A variety of genetic screens have been performed using mutagens, transposons, viruses, antisense morpholinos, and, more recently, gene editing. Transgenics are easily generated and these frequently exploit the model's transparency to label proteins, visualise processes or activate neurons via optogenetics in a spatio-temporal manner, as well as kill target cells with a laser. Transgenics are also useful for identifying donor and host cells after transplantation of tissue. Examples of all these methods are reviewed elsewhere [26].

Zebrafish embryos and larvae readily absorb chemicals in which they are bathed and this provides a quick and easy delivery route for chemicals. They are just 1-2 mm in length so can be housed in very small wells such as in a 96 or 384 well plate, therefore needing only small amounts of chemical, and making chemical treatments relatively cheap. These advantages have resulted in the use of zebrafish embryos are larvae for toxicological assessment and chemical screens, which is particularly useful when searching for new drugs. Although the delivery route is likely to be somewhat different from a final drug, and the zebrafish may not have yet developed its blood-brain barrier (which it does at 3 dpf), this is nevertheless a powerful method in drug discovery (reviewed in [30]).

As a vertebrate, the zebrafish is closer in evolutionary terms to humans than the fruit fly and other invertebrates, but less close than the mouse and other mammals. Indeed, the nervous system has a very similar plan throughout and homologous structures and connections can be identified, except for the lack of a cerebral cortex. However, zebrafish vision is cone-dominated and therefore considered to reflect the human situation better than the mouse [31] and this may be pertinent to NCL research. On the other hand, the ability of the zebrafish to regenerate tissues such as various parts of the retina, the spinal cord and the heart, is something many scientists are trying to understand to enable us to stimulate regeneration of human tissues.

The last 15 years has seen a huge increase in the number of diseases modelled in zebrafish, including those affecting the nervous system. Indeed, some models are turning out to be very useful as the effect of drugs in zebrafish is predictive of the effect in humans and this has led to drugs reaching clinical trials for human neurological diseases such as epilepsy [32, 33] and amyotrophic lateral sclerosis (ALS) [34, 35]. The generation of genetic models of disease can sometimes be hampered by genome duplications during evolution, meaning that there are often two zebrafish homologs for a human gene [36]. As we do not know what functions are retained or altered in each homolog, we frequently have to target both to generate a model. Quite often, antisense morpholino oligonucleotides (MO) are injected into the newly fertilised embryo to knock-down the target protein during the first few days of life. This frequently results in NCL-like phenotypes, but great care must be taken to

control for off-target effects and toxicity (guidelines found in [37]). Fortuitously, several large forward and reverse mutagenesis screens have resulted in mutations in NCL genes, and these are awaiting phenotyping to assess their suitability as disease models. To generate new mutations in NCL genes, gene-editing techniques are now predominantly used [38]. It is curious that not all loss-of-function mutations in *CLN* genes result in an NCL-like phenotype, and it remains to be seen if a compensatory mechanism is elicited in these mutant, but phenotypically normal, zebrafish. If this turns out to be the case, perhaps that compensatory mechanisms can be elicited in patients to provide a novel therapeutic strategy.

To date, acceptable zebrafish models for CLN2 and CLN10 diseases exist (Table 3), with both of these exhibiting embryonic and larval phenotypes reminiscent of NCL to varying degrees. Many other mutants have not yet been researched, and details of these can be found on the zebrafish database ZFIN (zfin.org). This suggests that the zebrafish is currently being under-utilised for NCL research.

2.4 Fruit fly

The fruit fly, *Drosophila melanogaster*, has been used as a model to study genetics, developmental biology and neurobiology for more than a century. The unparalleled genetic tools available in *Drosophila* allow for more detailed manipulation of genes, genotypes and phenotypes than in any other laboratory model and the short generation times, ease of handling and absence of licencing requirements permit these tools to be used on a genome-wide basis [39]. *Drosophila* has homologues of 75% of human disease-causing genes [40] and the ease of manipulating these genes has made flies a popular choice to model human diseases. They are particularly useful for monogenic inherited disorders where the *Drosophila* gene can be mutated to mirror the human condition, and in gain-of-function disorders, including forms of neurodegeneration such as Alzheimer's, Parkinson's and Huntington's diseases [41-43]. In addition, the *Drosophila* CNS has been used for many decades as a model system to understand aspects of neural development and function: from identifying the transcription factor networks that regulate neural specification; how components of the synapse function; to the pioneering of optogenetic tools to manipulate neural activity to map the circuits that control behaviour [44-46]. Despite the much smaller size and obvious anatomical differences between the mammalian and insect brains, many functions are orthologous, and the smaller size enables *in vivo* imaging. Like the mammalian brain, the fly brain is modular, with regionally-specific functions controlling complex behaviours, including learning and memory, movement and visual processing [47]. The major excitatory and inhibitory neurotransmitters used in the mammalian CNS are also used in the fly, along with numerous neuropeptides [48, 49]. The fly brain also has specialised glia with orthologous roles to mammalian glia, and a blood-brain-barrier isolating the CNS [50].

Drosophila has only a subset of the human CLN disease genes: the soluble lysosomal enzymes, *Ppt1* (CLN1), cathepsin D (*Drosophila* gene name = *cathD*) and cathepsin F (CG12163); the membrane proteins, *Cln3*, *Cln7* (CG8596) and ATP13A2 (*anne boleyn*); and cysteine string protein (*Csp*). Unusually, cysteine string protein had been studied extensively for its role in synaptic function prior to its identification of as a NCL disease locus. Of the remainder, *Ppt1* and *Cln3* are perhaps the best understood with both gain-of-function and loss-of-function studies reported for both genes. Recent single cell transcriptomics studies indicate that the lysosomal enzymes are

widely expressed within the CNS and present in most, potentially all, neurons. In contrast, the *Cln3* and *Cln7* genes are more restricted. Both genes are expressed primarily in glia, with *Cln3* also expressed in some neurons (see http://scope.aertslab.org/ for scRNA-seq data).

2.5 Nematode

Caenorhabditis elegans (*C. elegans*) is a small, non-parasitic nematode worm. In the wild, it feeds on bacteria in decomposing vegetable matter. In the laboratory, it is normally maintained on agar plates with bacteria (usually *E. coli*) added as a food source. Over 99% of worms are self-fertilising hermaphrodites, and a single hermaphrodite can produce around 300 eggs using its own sperm [51]. The ability of a single worm to generate large numbers of genetically identical offspring is very useful, as it enables stable maintenance of mutant strains without the need for mating. Nevertheless, rare males can be isolated and mated with hermaphrodites, which increases fecundity to around 1000 eggs per hermaphrodite. This ability to mate with males is important, as it enables genetic crossing for applications such as construction of double mutant strains. Development from an egg through four larval stages to the approximately 1 mm-long adult usually takes around 3 days at 20°C [52]. However, if food is scarce, worms arrest their development and enter an alternative larval form known as dauer. These dauer larvae can survive without eating for several months, resuming normal development once food becomes available. This is useful in the laboratory, as worms on agar plates can be left unattended for many weeks without fear of losing an important strain. Furthermore, *C. elegans* can withstand freezing and thawing, permitting the long-term maintenance of worms as frozen stocks. Adult lifespan is usually around 3 weeks under well-fed conditions, which facilitates the analysis of age-related phenotypes, such as adult onset forms of NCL.

These characteristics help to explain why *C. elegans* was adopted by Sydney Brenner as a new model organism in the 1960s. However, Brenner's main reason was that the extreme simplicity of *C. elegans* made it feasible to determine the complete structure of the nervous system [52]. Indeed, the *C. elegans* nervous system comprises just 302 neurons, out of a total of 959 cells in the adult hermaphrodite; whereas *Drosophila* contains around 100,000 neurons. *C. elegans* is unique among animals in that the position and developmental fate of every cell from zygote to adult is fully documented [53, 54], as are all of the synaptic connections made by its 302 neurons [55]. The animal is naturally transparent, and individual neurons can be visualised in freely moving animals via cell-specific promoters driving fluorescent reporter proteins such as GFP. This is particularly useful for studies of neurodegenerative diseases such as NCL, as it enables direct assessment of neuronal degeneration and death *in vivo* by visualising neuronal fluorescence as a readout [56]. Thousands of hermaphrodites can be stably maintained on agar plates without the need for mating, making *C. elegans* well suited to high-throughput genetic screens. Indeed, the unique ability to perform genome-wide knock down of protein expression via RNA interference (RNAi) simply by feeding the worms transformed versions of their standard laboratory food source, *E. coli*, is particularly powerful in this regard [57]. One drawback for the study of NCLs is that worm neurons are relatively resistant to RNAi compared to other tissues, but this issue can be addressed by using strains that are generally hypersensitive to RNAi [58] or exhibit enhanced RNAi specifically in neurons [59]. Finally, the extreme simplicity of the worm nervous system means there

are no ethical issues about animal suffering to be considered. Taken together, these various features of *C. elegans* explain why it has become one of the main model organisms used worldwide despite being developed comparatively recently.

2.6 Social amoeba

The social amoeba *Dictyostelium discoideum* has emerged as an excellent model organism for studying a variety of neurological disorders (e.g. Alzheimer's, Parkinson's and Huntington's Disease, epilepsy, etc) , including the NCLs [60-65]. Its 34 MB haploid genome, which is contained on six chromosomes, encodes approximately 12,500 proteins, is fully sequenced and annotated, and contains homologs of 11 of the 13 human NCL genes [62, 66, 67]. One of the main advantages of using *Dictyostelium* as a biomedical model system is its 24-hour life cycle that is comprised of distinct unicellular and multicellular phases [68] (Fig. 1). This allows researchers to study the effects of gene-deficiency on conserved cellular and developmental processes (e.g., cell movement, cell differentiation, intracellular trafficking, autophagy, etc.) within the context of a whole organism [68]. In the presence of nutrients, *Dictyostelium* grows as single cells, dividing by mitosis and obtaining nutrients by endocytosis [68] (Fig. 1). Nutrient depletion prompts a 24-hour developmental program that consists of a sequence of well-defined events. Upon starvation, cells secrete cAMP, which acts as a chemoattractant causing cells to aggregate into multicellular mounds. Mounds then form motile slugs (or pseudoplasmodia) composed of primarily two cells types, pre-stalk and pre-spore. Terminal differentiation of these cells results in the formation of a fruiting body comprised of a mass of dormant spores that are supported above the surface by a slender stalk. In nature, the spores are dispersed and when conditions are favourable (e.g., food source available), they germinate to form single cells, thus re-starting the life cycle.

Dictyostelium cells grow rapidly in the lab at room temperature and can be cultured inexpensively in liquid medium (8-12 hour doubling time) or on agar plates with bacteria (3-4 hour doubling time) [69]. Due to their fast doubling time, cells can be harvested in large numbers and used for a variety of cellular, developmental, and biochemical assays [68]. *Dictyostelium* is amenable to genetic manipulation (RNA interference, restriction enzyme-mediated integration mutagenesis, site-directed mutagenesis, multiple gene knockout via homologous recombination or CRISPR/Cas9-mediated targeting) and a variety of expression constructs have been generated to facilitate studies on protein localization and function [70-76]. In addition, several proteomic analyses have been performed in *Dictyostelium* to reveal proteins that are secreted during development, proteins that are contained within the extracellular matrix (ECM), and proteins that localize to the macropinocytic pathway [77-80]. These analyses show that 10 of the 11 NCL protein homologs are present in the macropinocytic pathway and 5 of the 11 homologs are secreted [77, 78, 80]. Importantly, these results are consistent with results from mammalian models of Batten disease that link the NCL proteins to a shared or convergent biological pathway [81]. Finally, there are many resources available to *Dictyostelium* researchers including dictyBase (the model organism database for *Dictyostelium*), dictyExpress (web application that compiles data from over 1,000 *Dictyostelium* microarrays and RNA-seq experiments of wild-type and mutant cell lines), and the Dicty Stock Center

at Northwestern University (central repository for *Dictyostelium* cell lines and expression constructs) [66, 82, 83].

Despite the evolutionary time gap between *Dictyostelium* and human, there are many advantages of using this organism as a model system for biomedical research. Since many human diseases, particularly those affecting the nervous system, share common cellular features such as storage body accumulation, mitochondrial dysfunction, and the accumulation of autophagic vacuoles, the study of *Dictyostelium* allows for the examination of these and other relevant processes in an organism with measurable phenotypic outcomes [60, 84]. Due to the metazoan-like behaviour of *Dictyostelium* cells, findings from this organism are highly translatable to more complex eukaryotic systems. Finally, the biology underlying the life cycle of *Dictyostelium* has been studied for more than 80 years, which has allowed the research community to reveal key components of the signal transduction pathways that regulate the growth and development of the organism.

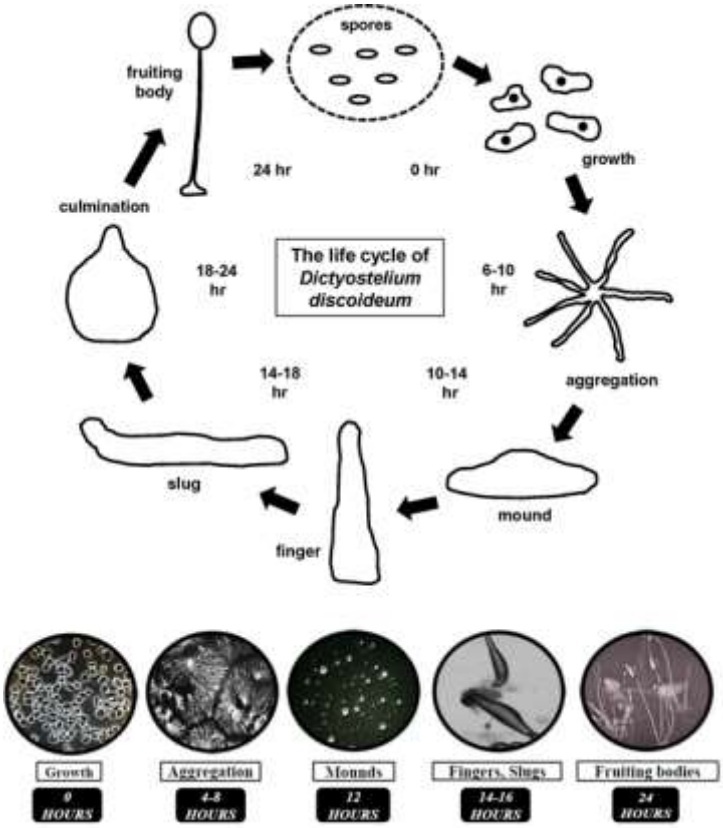


Figure 1. The life cycle of *Dictyostelium discoideum*. During growth, single cells feed on bacteria and divide by mitosis. Upon starvation, cells chemotactically aggregate to form multicellular mounds. Mounds then form fingers, which fall on the surface to generate motile slugs (also known as pseudoplasmodia). During culmination, terminal differentiation of pre-stalk and pre-spore cells forms fruiting bodies. Each fruiting body is composed of a slender stalk that supports a mass of dormant spores. When environmental conditions are favourable (e.g., food source available), the spores germinate allowing the cells to restart the life cycle. Top panel was previously published in [68]. Bottom panel was previously published in [85].

3. CLN1 disease, caused by mutations in Palmitoyl protein thioesterase (PPT1)

Homozygous mutations in the lysosomal enzyme Palmitoyl protein thioesterase 1 (PPT1) cause CLN1 disease.

3.1 Large animals

To date mutations in *CLN1* have been confirmed in miniature Dachshunds (*CLN1:c.736-737insC*; [86] and the Cane Corso (*CLN1:c.124 +1G>A,* a splice site mutation; [87]). Both founders exhibited symptoms around 8 months of age with progressive blindness, ataxia and lethargy. To our knowledge neither of these dogs have been established as a research colony.

3.2 Rodents

The mouse homolog of PPT1, a 306 amino acid protein encoded on chromosome 4, shares 85% identity with human PPT1 (CLUSTALW (2.1) Multiple Sequence Alignment on Ensembl (www.ensembl.org)). For both mice and rats, PPT1 is highly expressed in the developing brain and eyes [88-91]. Particularly, PPT1 is expressed in the synaptic compartments of mouse neurons [90, 92-94], indicating its potential involvement in synaptic development or function.

Genetically modified mouse models with PPT1 deficiency have been developed to model CLN1 disease. Two *Ppt1* null mouse lines were generated independently by disruption of either exon 9 or 4 of the *Ppt1* gene [95, 96]. Characterising studies showed typical NCL-like pathology in various CNS regions, including neuronal autofluorescent storage material accumulation, astrocytosis, microgliosis and neurodegeneration, with changes in the cerebellum and thalamus earlier than in the cerebral cortex [95-99]. In the visual system, apart from pathology of the visual nuclei in the thalamus, *Ppt1* null mice also show retinal ganglion cell and photoreceptor cell loss as well as immune cell infiltration into the optic nerve [98, 100, 101]. *Ppt1* null mice exhibit phenotypes typical of CLN1 disease, including progressive motor abnormalities, seizures, vision loss and a markedly shortened lifespan [95, 96, 102]. Khaibullina *et al.* found the *Ppt1* mouse also displayed age-dependent impairment of thermoregulation, which is also observed in a group of CLN1 patients [103, 104]. More recently, another two mouse lines modelling CLN1 disease have been generated by knocking in the most common CLN1 disease-causing nonsense mutation R151X, both of which recapitulated the phenotype and pathology hallmarks of CLN1 disease [105, 106]. These knock-in mice offer more options for *in vivo* studies of CLN1 disease, and particularly, provide a more disease-relevant model for testing nonsense suppression therapies.

Mouse models of CLN1 disease are invaluable tools for neurobiological studies especially at pre-symptomatic stages of the disease, which could provide important information on PPT1 functions and mechanisms underlying CLN1 disease. A proteomic analysis of the *Ppt1* null mouse has revealed protein profile changes at different stages, and highlighted dysregulated protein trafficking and mitochondrial function starting pre-symptomatically, and changes of synaptic and myelin proteins at early-symptomatic stages [107]. It provides clues for the biological pathways initially affected in the disease and different aspects of PPT1 functions. Several studies using the *Ppt1* null mouse combined with *in vitro* culture systems have revealed PPT1

functions in the lysosome, such as contribution of PPT1 on maintaining lysosomal degradative functions by affecting cathepsin D maturation [108] and on lysosomal normal acidification by promoting vacuolar ATPase assembly [109]. Moreover, given that PPT1 is observed in the synaptic compartments of neurons, PPT1 functions in synapses could be of particular importance in the CNS. Indeed, the *Ppt1* mouse shows impaired recycling of synaptic vesicle proteins and decreased synaptic vesicle pools in the presynaptic terminal, which is caused by loss of the PPT1 depalmitoylating activity [94]. Such synaptic changes may represent a potential mechanism underlying CLN1 disease progression, independent of PPT1-related lysosomal dysfuctions. Additionally, astrocytes appear to contribute to the pathogenesis of CLN1 disease. Characterization of *Ppt1* null mice showing astrocytosis occurring prior to neuronal loss and microgliosis in the thalamus, cerebellum and cerebral cortex suggests astrocyte alteration as an early pathological change rather than a secondary response to neurodegeneration [98, 99, 110]. *In vitro* evidence shows PPT1 deficiency in astrocytes results in an exacerbated reactive state that is more neurotoxic, but exact contribution of such changes to CLN1 disease still needs further validation *in vivo* [111].

Full spectrum characterisation of the *Ppt1* null mouse makes it a good model to test therapies. For CLN1 disease, various therapeutic strategies have been developed and studied pre-clinically in the *Ppt1* null mouse models. Since PPT1 is a soluble lysosomal enzyme, enzyme replacement has been suggested. A recombinant PPT1 has been tested in the *Ppt1* null mouse model [112, 113]. Chronic intravenous delivery from birth and short-term intrathecal delivery at 6 weeks of age (pre-symptomatic) both significantly delayed the behavioural phenotype development and increased the lifespan; however, the improvement is limited, probably due to lack of persistent high PPT1 activity in the CNS. NtBuHA, a non-toxic molecule crossing the blood-brain barrier and mimicking the thioesterase activity of PPT1, was also tested with the *Ppt1* mouse model and showed improvement of several PPT1-related lysosomal functions such as restoring vacuolar ATPase activity and cathepsin D activity [108, 109]. Also, pathology including autofluorescent storage material accumulation, neuronal loss, astrocytosis and motor abnormality were improved with 3 months treatment starting pre-symptomatically, but still, lifespan was only slightly improved [114]. Similarly, since the mice only received 3 months treatment, the issue of achieving long-term enzyme activity needs to be resolved. Moreover, gene therapy is considered as an attractive strategy for various NCLs. For CLN1 disease, brain-targeted gene therapy via recombinant AAV vectors has also been performed on the *Ppt1* null mouse. Brain pathology and motor function impairment were significantly decreased in a few pre-clinical studies with AAV gene therapy intracranially delivered to newborn mice [115-118]. Although seizure frequency and lifespan were not obviously changed in a study published in 2006 [116], improvement was shown in other later studies using a much higher dose of viral vectors [115, 118]; however, the lifespan was still much shorter than wild-type mice. Combining gene therapy with systemic administration of either a PPT1 mimetic or an anti-inflammatory drug did not produce a large synergistic effect [115, 118]. Interestingly, coupled intracranial and intrathecal AAV gene transfer was found to rescue the pathology throughout the CNS including the spinal cord and largely increase the lifespan to around 19 months, suggesting the clinical significance of more widespread CNS treatment for CLN1 disease [119]. Therefore, in future, novel approaches could be further studied on CLN1 mouse models in order to achieve more persistent and broader distribution of PPT1 correction.

3.3 Zebrafish

The zebrafish has one homolog of PPT1. The surrounding synteny is preserved, as are the splice sites. The zebrafish gene encodes a predicted 303 amino acid protein with 65% identity with humans (CLUSTAL W (1.81) multiple sequence alignment on Ensembl (www.ensembl.org)). Zebrafish Ppt1 is expected to be found in all tissues. The mRNA is highly expressed from fertilisation to gastrulation (https://www.ebi.ac.uk/gxa/home), and then at a lower level up to 5 days post fertilisation (dpf). Expression of the mRNA appears to be higher in the developing brain [120]. The protein has been reported as found in synaptosome fractions in the adult zebrafish central synapses, in contrast to both synaptosome and post-synaptic density fractions in mice [121]. To date, there are no publications showing phenotypes caused by mutation in zebrafish Ppt1. Two nonsense mutations do exist (Table 3), which could be examined for NCL-like phenotypes.

3.4 Fruit fly

CLN1 disease was the first NCL to be modelled in the fruit fly [122]. *Drosophila* Ppt1 protein shares greater than 55% identity with the human protein and the gene is widely expressed throughout the development stages of the fly and is maintained in adults. Recent single cell transcriptomics indicates that Ppt1 is expressed in both neurons and glia in the adult CNS but expression is not detectable at high levels in all neurons. This is consistent with similar scRNA-seq data from human CNS cells (see atlas at http://scope.aertslab.org/). The *Ppt1* gene lies on the X chromosome and is uncovered by a small deficiency, Df(1)446-20, that deletes three genes. Male flies hemizygous for this deficiency or females trans-heterozygous for the deficiency and either of two point mutations in the *Ppt1* gene accumulate autofluorescent storage material in lysosomes. The material differs somewhat from the classic granular deposits seen in CLN1 disease and instead are more laminar in nature [122]. Interestingly, *Ppt1* deficient flies show little or no sign of neurodegeneration despite quite dramatically reduced lifespan [122].

A major advantage of using *Drosophila* to study aspects of human disease is the ability to use unbiased, genome-wide genetic screens to provide an insight into the cellular pathways and genes involved in the biology of the disease. This approach has been used to screen for modifiers of a *Ppt1* overexpression phenotype in the developing fly eye which disrupts the regular array of ommatidia [123, 124]. The genes emerging from these screens pointed strongly to a role for Ppt1 outside of the lysosome in regulating endocytosis, confirmed by follow-up experiments that revealed defects in endocytosis in Ppt1 deficient cells [124]. Interactions were also regulating pre-synaptic development via retrograde signalling from the post-synaptic cell [123, 124]. These intriguing interactions led to experiments that exploited a second strength of *Drosophila* – as a model system for neurobiology – to look for roles of Ppt1 in neural function.

Maintaining normal synaptic activity is highly dependent on endocytosis to retrieve synaptic vesicle membrane. Thus, blocking endocytosis, for instance with the temperature-sensitive dynamin mutation *shibire*^{ts}, leads to rapid paralysis when flies are shifted to the restrictive temperature. Flies carrying both the *shibire*^{ts} and loss-of-function *Ppt1* point mutations, became paralysed far more quickly than *shibire*^{ts} flies

alone suggesting endocytosis is further compromised when Ppt1 function is missing [125]. Synaptic vesicle transmission is also dependent on a palmitoylation cycle of key proteins, including SNAP, VAMP and syntaxin [126]. This suggests Ppt1, as a de-palmitoylating enzyme, may be involved in the regulation of synaptic transmission. Searching for such a role, Korey and colleagues examined synaptic vesicle release at the *Drosophila* larval neuromuscular junction, an accessible glutamatergic synapse that is used extensively as a model of mammalian excitatory synapses. In *Ppt1* mutants, synaptic vesicles were released spontaneously at an increased rate but, importantly, the amplitude of the miniature evoked junction potentials (EJPs) recorded from the post-synaptic cell was unaltered [125]. The mini EJP amplitude is dependent on the quantal content of the vesicles and the density and organisation of the post-synaptic glutamate receptors and, hence, these are likely to be largely unaffected by loss of Ppt1 function. The overall development of the larval neuromuscular junction was also unaffected, suggesting Ppt1 is not required for the BMP-based retrograde signalling[125]. This is in contrast to mutations in other lysosomal storage disorder proteins, including Spinster and Trpml, where mutations do result in clear developmental deficits at the neuromuscular junction [127, 128]. Still outstanding is to identify whether the *Drosophila* Ppt1 is present at synapses in addition to its principal localisation in lysosomes. However, Ppt1 has been detected in synaptosomes from human brain material and co-localises with the synaptic vesicle protein, synaptophysin, in cultured neurons [94]. Moreover, the levels of synaptic vesicle-associated proteins and synaptic vesicle trafficking are altered in brain material from CLN1 disease patients and form Ppt1 KO mice. The *Drosophila* electrophysiology studies are consistent with these mammalian findings. Viewed together, these studies argue that altered neuronal physiology results directly from loss of Ppt1 function in the pre-synaptic compartment and that this contributes to CLN1 disease, potentially independently of effects on lysosomal function.

3.5 Nematode

Although mutations in *PPT1* cause very severe phenotypes and death at young ages in humans and mammalian models, mutation or RNAi knockdown of the *C. elegans* orthologue, *ppt-1*, has surprisingly little effect [129, 130]. A variety of phenotypes have been examined, including size, morphology, fecundity, lifespan, locomotion, age-dependent motility, mechanosensation, autofluorescence and lipid accumulation. Some very mild effects on individual phenotypes were observed for specific mutant alleles or RNAi knockdown. For example, *gk131* deletion mutants exhibited a small increase in locomotion and maximum lifespan, whereas *ppt-1* RNAi showed a decreased median lifespan using an RNAi hypersensitive strain [130]. However, no consistency was seen across the different mutant alleles analysed, suggesting that the observed effects were unrelated to loss of PPT-1 function. It is unlikely that the mild effects reported are due to inefficient impairment of PPT-1 protein function, as the *gk131, gk134, gk139* and *gk140* alleles studied are insertion/deletion mutants that are predicted to produce non-functional enzymes (Table 4). Furthermore, *gk139* has been shown experimentally to be a null allele and lysates from *gk139* strains display greatly reduced PPT-1 enzyme activity [129]. Interestingly, electron microscopy analysis of *gk139* mutants exhibited altered mitochondrial morphology in young worms, which may be related to the slight developmental and reproductive delay observed in this strain and the *gk140* mutant [129]. Although this may be relevant to mitochondrial dysfunction in CLN1 disease, it is difficult to understand why the reported

mitochondrial abnormalities in *gk131* had no effect on lifespan [129], a phenotype that is known to be strongly affected by mitochondrial dysfunction. One potential explanation for why mutation and/or RNAi knockdown of *ppt-1* has such a minor effect in *C. elegans* is redundancy with another depalmitoylating enzyme. The *C. elegans* genome encodes only one other predicted palmitoylprotein thioesterase: the cytoplasmic enzyme, ATH-1 [130]. However, double RNAi knockdown of both *ppt-1* and *ath-1* together, or performing *ppt-1* RNAi in an *ath-1* mutant background also failed to produce strong phenotypes [130]. Presumably, an additional, as-yet-unknown mechanism in *C. elegans* compensates for the loss of PPT-1 function. Identification of this putative redundant mechanism could have therapeutic implications, as it might represent an alternative drug target for CLN1 disease. Additionally, interfering with this compensatory mechanism in a *ppt-1* mutant genetic background might then create the required strong phenotype where the real power of *C. elegans* in applications such as high-throughput genetic and pharmacological screens could be used to good effect. For the moment, however, the very mild phenotypes exhibited by worm *ppt-1* mutants make modelling CLN1 disease in *C. elegans* challenging.

3.6 Social amoeba

The *Dictyostelium* homolog of human PPT1 is a 303 amino acid, 34 kDa protein (Ppt1) encoded by the *ppt1* gene. Within a 273 amino acid region of similarity between Ppt1 and PPT1, 46% of the amino acids are conserved and 69% are positive matches [62]. Like human PPT1, the *Dictyostelium* homolog contains a signal peptide for secretion, indicating that it may function extracellularly [62]. Not surprisingly, Ppt1 has been detected in conditioned buffer from developing *Dictyostelium* cells [77]. In addition, like many of the other NCL protein homologs in *Dictyostelium*, Ppt1 is present in the macropinocytic pathway [80]. The expression of *ppt1* increases dramatically upon starvation and peaks during the early stages of multicellular development [83]. Expression then decreases dramatically during the mid-to-late stages of development when cells terminally differentiate to form fruiting bodies [83]. This expression profile suggests that Ppt1 may play a role in cAMP-mediated chemotaxis and aggregation. While a knockout mutant has not yet been generated to study the function of Ppt1 in *Dictyostelium*, previous work suggests that the protein may play a role in phagocytosis, which is consistent with data from mouse models linking the function of PPT1 to phagocyte infiltration following neuronal death [131, 132].

4. CLN2 disease

Homozygous mutations in the lysosomal enzyme Tri-peptidyl peptidase 1 (TPP1) cause CLN2 disease.

4.1 Large animals

4.1.1 CLN2 disease in the Dachshund dog

Mutations in canine CLN2 were first reported in the miniature longhaired Dachshund in 2006. These dogs present with cognitive impairments at 9 months old, and ataxia and visual deficits by 10 months of age. The CLN2 dog mutation (c.325delC) causes

a frameshift and premature stop codon. TPP1 enzyme activity is undetectable in brain tissues in these animals [27].

Disease progression has since been carefully characterised, using a range of biomarkers and behavioural analyses. *CLN2-/-* dogs display clinical signs by 5 months of age, progressing from hindlimb impairments to ataxia, loss of menace responses at 6.5 months, eye involvement by 8.7 months and tremor at around 9 months, with end stage disease by 10-11 months. Cognitive decline is apparent using T-maze testing at 7-9 months. These data, together with pathological findings, provide the basis for evaluating the efficacy of several therapeutic approaches including enzyme replacement therapy (ERT), mesenchymal stem cell transplantation, drug therapy and gene therapy.

CLN2 disease is the first NCL to be treated clinically with enzyme replacement therapy (ERT) [133] and much of the pre-clinical work has been done in the *CLN2-/-* dogs [134-136]), validating previous mouse work [137, 138] and safety testing in dogs and monkeys [136, 139]. Catheterisation of dogs at 2 months of age was followed 2 weeks later, and fortnightly thereafter, with infusion of recombinant human TPP1. Affected and wildtype dogs (n=3/group) were assigned to one of three doses of enzyme or vehicle. Two dogs on the highest dose had adverse events due to the catheter/treatment. All dogs underwent neurological and physical examinations, cognitive testing and magnetic resonance imaging (MRI). Development of neurological deficits were delayed in the higher dose groups compared to vehicle and cognitive function and brain volumes were preserved and survival significantly increased. Human clinical trials commenced in 2013 and both the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have now approved ceroliponase alfa (Brineura) for clinical use in CLN2 patients [133].

The CLN2 dogs have also provided key data in the move towards gene therapy for CLN2 disease. Intracerebroventricular (ICV) infusion of canine AAV2-*TPP1* at 3 months of age (pre-symptomatic) resulted in predominantly ependymal transduction and spread of TPP1 protein throughout the brain and spinal cord [140]. Notably, the original studies in mice demonstrating AAV4 was the optimal serotype for ependymal transduction [141] failed to replicate in the dog, suggesting translation of animal data needs to be carefully considered in non-human primates. In addition, immunosuppression using mycophenolate mofetil and cyclosporine was required to prevent the generation of anti-TPP1 antibodies and loss of TPP1 activity in null dogs. Most importantly, AAV2-TPP1 administration to pre-symptomatic dogs delayed disease onset and extended lifespan, however the dogs did eventually succumb to disease.
Although NCLs are primarily neurological diseases, there is increasing awareness of peripheral pathology, which have become more apparent as the neurological deficits are treated and lifespan increases. In *CLN2-/-* dogs, general blood biomarkers of tissue damage including cardiac troponin, alanine aminotransferase and creatine kinase increase over time, even in treated dogs. Electrocardiography (ECG) parameters were also significantly impaired in *CLN2-/-* dogs and not restored by ICV AAV2-*TPP1*. The authors suggested combined peripheral and central nervous system (CNS) gene transfer may be required [142].

Alternatively, newer generation vectors that provide both systemic and CNS transduction from systemic administration may provide body-wide therapy. AAV9-*TPP1* has recently been given orphan drug designation from the FDA, providing a

fast-track to clinical trial (https://regenxbio.gcs-web.com/news-releases/news-release-details/fda-grants-orphan-drug-designation-rgx-181-gene-therapy).

Another key organ for treatment in most NCLs is the eye. In *CLN2-/-* dogs, there is progressive loss of electroretinogram (ERG) activity with no recordable scotopic or photopic activity by 12 months of age [143]. ICV injection with AAV2-*TPP1* preserved the retinal ganglion cells, however the remainder of the retina eventually deteriorated and there was no preservation of ERGs in the treated affected dogs. [144].

An alternative treatment approach used autologous bone marrow derived mesenchymal stem cells as a vehicle to deliver AAV2-*TPP1* to the eye. AAV2-TPP1 transduced stem cells were transferred into the vitreous of one eye in each of three 14-week-old dogs [145]. Treated eyes had preserved retinal structure and ERG responses out to 10.5 months of age (end of study).

Combined, these studies suggest that no one treatment will be sufficient and it is likely that a combination approach will be required for treatment of central and systemic pathologies.

Translation of preclinical gene therapy studies in the mouse model of CLN2 disease, required safety studies to be completed in non-human primates. African green monkeys were used to demonstrate appropriate transduction and spread of TPP-1 in the larger monkey brain and that safety and toxicity data was obtained [146].

4.2 Rodents

The mouse homolog of TPP1, a 562 amino acid protein encoded on chromosome 7, shares 88% identity with human TPP1 (CLUSTALW (2.1) Multiple Sequence Alignment on Ensembl (www.ensembl.org)). *Tpp1* is highly expressed in the developing mouse, especially in the regions of high neurogenesis activity [147]. The expression pattern of TPP1 activity during development is slightly different between humans and mice. In the mouse CNS there is a persistent increase of TPP1 activity during the infantile and juvenile developing stages, which reaches the highest level at adulthood; while in the human brain the adult TPP1 activity level has been reached by 2-4 years of age, suggesting potential differences in TPP1 function in the mouse compared to the human [148].

A *Tpp1* knockout mouse model has been generated to model CLN2 disease [29]. The mouse exhibits tremors starting from 7 weeks of age followed by progressive motor dysfunctions and a severely shortened lifespan (3-6 months) [29]. NCL-like neuropathology such as autofluorescent storage material accumulation and astrocytosis is observed, especially in the cerebellum [29, 138]. Cerebral and cerebellar neurodegeneration and axonal degeneration are also observed, but the pathology in the mouse cerebral cortex appears to be milder than in the human patients. Retinal degeneration is not observed in this mouse model. Despite the differences between the *Tpp1* knockout mouse and human CLN2 disease, it still recapitulates many aspects of CLN2 disease and could be a useful tool for studying CLN2 disease, especially for testing potential therapies. Modification of this knockout model generated a line expressing *Tpp1* with a missense mutation R446H that resulted in low levels (around 6%) of TPP1 activity in the brain [149]. This mouse line shows that 6% of normal TPP1 activity is capable of producing dramatic improvements and extension of lifespan, which provides clues for the minimal TPP1 levels that should

be achieved to produce proper therapeutic benefits. More recently, a knock-in mouse model harbouring R207X, equivalent to the most common CLN2 disease nonsense mutation (p.R208X), has been generated, which allows for assessing antisense oligonucleotides and nonsense suppression therapies [150]. This knock-in model exhibits severely decreased *Tpp1* expression and TPP1 activity in the brain and visceral organs. Lysosomal accumulation of mitochondrial ATP synthase subunit C and astrocytosis are observed in the cerebral cortex of the knock-in model. The knock-in mouse displays tremors and motor function impairment from 3 months of age and a similar lifespan to the *Tpp1* knockout mouse model.

Pre-clinical studies with potential therapies for CLN2 disease have been widely performed. Brain-targeted gene therapy using the AAVrh.10 vector with *TPP1* was investigated in *Tpp1* knockout mice and showed promising improvements of neuropathology and behavioural performance and lifespan extension [137, 151, 152]. Notably, time of treatment appeared to have marked impacts on the therapeutic outcomes; pre-symptomatic delivery of gene therapy produced significantly better motor function improvement and particularly extended lifespan (around 11-12 months), with possible reasons being early intervention before disease onset and/or wider distribution of transgene in the developing brain [152, 153]. Systemic delivery of viral vectors harbouring *TPP1* has also been studied. Intra-arterial AAV-TPP1 injection accompanied by pre-injection of mannitol (a blood-brain barrier permeabilizer) led to widespread transgene distribution in the mouse brain, which provided a novel gene transfer approach for CLN2 disease and other CNS diseases [154]. Apart from gene therapy, enzyme replacement therapy (ERT) is another attractive therapeutic strategy for CLN2 disease, given the establishment of a recombinant human TPP1 pro-enzyme that could be endocytosed and activated in the lysosome [155]. CNS-targeted ERT with this recombinant TPP1 pro-enzyme showed improvements of neuropathology and behavioural performance with a slightly extended lifespan in the knockout mouse model [138, 156], which was then tested in a CLN2 canine model and a small group of human patients and produced significant treatment benefits [133, 135, 136]. Furthermore, systemic (intravenous) delivery of recombinant human TPP1 produced dose-dependent brain TPP1 activity increase, which reached 10% of the wildtype level with a 2mg-dose injection [157]. As discussed earlier, achievement of 6% of the normal brain TPP1 level is capable of producing significant therapeutic benefits; so intravenous delivery of high dose TPP1 could be an alternative therapeutic approach for CLN2 disease, particularly considering that human patients receiving CNS-targeted ERT via intraventricular infusion show risk of serious infusion-device-related complications [133] and that peripheral system could also be affected by TPP1 deficiency, systemic delivery of TPP1 might be a more appropriate therapeutic strategy for CLN2 disease, while potential risk of immune-related adverse effects that could be induced by systemic ERT should also be investigated. Combined intravenous injection of TPP1 and K16ApoE (a modified ApoE peptide) to the knockout model facilitated TPP1 distribution throughout the brain without damaging the blood-brain barrier [158]. However, *in vitro* assays revealed a dose-dependent cytotoxicity of K16ApoE, which should be investigated before such an approach is considered clinically. Additionally, a recent study showed that orally administrated gemfibrozil (a lipid lowering drug) to the *Tpp1* knockout mouse displayed a moderate but significant improvement of the disease phenotype, indicating a potential alternative and more accessible option for CLN2 disease treatment [159].

4.3 Zebrafish

The sole homolog of TPP1 in zebrafish, called Tpp1, is 582 amino acids and has 57% identity with its human homolog at the protein level (CLUSTAL W (1.81) multiple sequence alignment on Ensembl (www.ensembl.org)). The exon/intron boundaries are well conserved but there is no synteny here between the zebrafish and human chromosomes around Tpp1. mRNA expression is predominantly in the brain of the developing zebrafish [120] and it has been shown in the retina at 48 hpf [28].

The zebrafish *tpp1*^{sa11} homozygous mutant was published as a model of human CLN2 disease [28]. A further mutant, *tpp1*^{hu3587} was homozygous viable, with trans-heterozygotes demonstrating a mild and variable phenotype. Injection of antisense morpholinos designed to knock-down Tpp1 produced a similar phenotype to that seen in *tpp1*^{sa11} homozygotes. The *tpp1*^{sa11} model has been used most as the phenotype progresses very fast: it is first obvious as a small eye at 48 hours post-fertilisation (hpf), and mortality occurs by 7 days-post-fertilisation (dpf). With a variety of phenotypes and pathologies relevant to various aspects of the disease, the model can be used to study as many aspects of the disease as needed. However, there is an absence of auto-fluorescence – perhaps due to the fast disease progressing - and electron microscopy has not yet been performed to visualise the membrane profiles.

This model has been used to test premature-stop codon read-through drugs, demonstrating those tested to have little therapeutic potential ([160] and reviewed in [11]). Further conference abstracts suggest that it may be a suitable model for testing anti-epileptics as *tpp1*^{sa11} homozygotes treated with valproic acid have reduced epileptiform activity and better survival than those treated with pentobarbitone [161], and that a novel anti-epileptic drug was found through phenotypic screening of several hundred compounds [162]. Finally, abrogating inflammation does not improve the *tpp1*^{sa11} phenotype, as shown through genetically-induced microglia ablation and the use of ibuprofen and prednisolone [163].

Altogether, the information suggests that this model has potential for finding and assessing new compound treatments, though it is likely that compounds would need to be tested on other systems to assess the likelihood that the results can be translated to humans.

4.4 Fruit fly

Drosophila does not have a clear homologue of the *TPP1* gene.

4.5 Nematode

C. elegans does not have a clear homologue of the *TPP1* gene.

4.6 Social amoeba

The *Dictyostelium* genome contains six genes (*tpp1A*, *tpp1B*, *tpp1C*, *tpp1D*, *tpp1E*, and *tpp1F*) that encode proteins sharing sequence similarity with human TPP1 [164, 165]. Of the six genes, *tpp1B* is the most highly expressed during the life cycle, followed by *tpp1F* and *tpp1A* [83]. *tpp1C*, *tpp1D*, and *tpp1E* are all expressed at

relatively lower amounts [83]. *tpp1A* was the first homolog to be identified and characterized in *Dictyostelium* [164]. *tpp1A* encodes a 600 amino acid, 67 kDa protein (Tpp1A). Within a 552 amino acid region of similarity between Tpp1A and TPP1, 36% of the amino acids are conserved and 52% are positive matches [62]. Expression of *tpp1A* is low during growth but increases dramatically upon the onset of aggregation [83]. Expression peaks after 16 hours of development when the multicellular slug forms and then decreases dramatically during fruiting body formation [83]. Like human TPP1, Tpp1A localizes to the lysosome [164, 166]. Loss of *tpp1A* reduces Tpp1 activity in *Dictyostelium* and causes an accumulation of autofluorescent storage material in starved cells [164]. *tpp1A*-deficient cells are also less viable than wild-type cells and display impaired growth in autophagy-stimulating media suggesting that Tpp1A plays a role in regulating autophagy [164]. Importantly, autophagy defects have also been reported in fibroblasts obtained from CLN2 disease patients [167]. During the mid-to-late stages of development, loss of *tpp1A* causes cells to develop precociously and affects spore formation, suggesting that Tpp1A may also regulate cell differentiation [164]. While enzyme replacement therapy is currently being used to treat patients with CLN2 disease, alternate indirect approaches may also prove to be effective therapeutic options [3]. With this in mind, work in *Dictyostelium* used restriction enzyme-mediated integration- (REMI) mutagenesis to identify *stpA* (suppressor of Tpp1A) as a second site suppressor of *tpp1A*-deficiency [164]. StpA shares some similarity with oxysterol-binding proteins, which function in lipid metabolism and transport [168]. Intriguingly, altered lipid homeostasis has been linked to the NCLs [169, 170]. Thus, the discovery of a second site suppressor in *Dictyostelium* has set the stage for future work to study second site suppressors of loss-of-function mutations in human *TPP1*.

In addition to Tpp1A, the localizations and functions of Tpp1B and Tpp1F have also been studied in *Dictyostelium*. Tpp1B is a 598 amino acid, 65 kDa protein. Within a 589 amino acid region of similarity between Tpp1B and TPP1, 31% of the amino acids are conserved and 45% are positive matches. Tpp1F is a 702 amino acid, 76 kDa protein. Within a 369 amino acid region of similarity between Tpp1F and TPP1, 28% of the amino acids are conserved and 44% are positive matches. Unlike Tpp1A, both Tpp1B and Tpp1F localize to the macropinocytic pathway and are secreted during development [77, 78, 80]. Also, comparing the expression profiles of *tpp1B* and *tpp1F* with *tpp1A* suggests that the proteins function at different points during the life cycle. In contrast to *tpp1A*, *tpp1B* and *tpp1F* are highly expressed during growth [83]. Upon starvation, the expression of both genes decreases dramatically reaching their lowest levels after 8-12 hours of development [83]. Expression then remains low during the mid-to-late stages of development [83]. In contrast, *tpp1A* expression increases dramatically upon starvation, and remains high during all stages of multicellular development [83]. These findings indicate that Tpp1B and Tpp1F may be the dominant Tpp1 proteins during growth, while Tpp1A may be the dominant Tpp1 protein during development. Recent work has shown that Tpp1B and Tpp1F bind the Golgi pH regulator (GPHR), which is a transmembrane anion channel that acidifies compartments of the Golgi complex and regulates growth and the later stages of multicellular development [165, 171, 172]. This finding should fuel research in human models of CLN2 disease to further explore the possible functional interaction between TPP1 and the GPHR. In addition to the Golgi complex, Tpp1F also localizes to the endoplasmic reticulum (ER) and V-ATPase-positive vesicles [165]. Tpp1F has serine protease activity, but loss of *tpp1F* has no obvious effects on growth or development, which could be attributed to the activities of the other Tpp1 proteins in

Dictyostelium [165]. Unlike *tpp1A* and *tpp1F*, a *tpp1B* knockout mutant has not yet been generated. Therefore, future research that generates knockout models for the other *tpp1* genes in *Dictyostelium* is highly likely to reveal their precise roles in regulating the growth and development of the organism.

5. CLN3 disease

Homozygous mutations in the transmembrane protein CLN3 (Battenin) cause CLN3 disease.

5.1 Large animals

5.1.1 CLN3 disease in pigs

No large animal models for CLN3 disease have been identified. Using gene technology, a pig model was reported at by the Weimer and Pearce laboratories [16] that will provide a powerful model for future screening of therapies, complementing current animal and cellular models.

5.2 Rodents

The mouse homolog of CLN3 shares 86% identity with human CLN3 (CLUSTALW (2.1) Multiple Sequence Alignment on Ensembl (www.ensembl.org)). CLN3 is ubiquitously expressed in various mouse tissues including the CNS. Within the mouse CNS, *Cln3* is highly expressed in various brain regions at embryonic stages; however, from the perinatal stage *Cln3* is constantly down-regulated and in the adult brain shows dramatically low expression levels [173, 174]. *In vitro* subcellular localization analysis suggests that CLN3 is observed in both the soma and neurites of primary neurons and mainly localized to late endo-lysosomal compartments [174]. $CLN3^{\Delta ex7/8}$, mimicking the most common CLN3 disease genetic mutant form, results in retained CLN3 in the ER, suggesting impaired CLN3 trafficking [174].

So far, there have been four CLN3 deficient mouse models generated [173, 175-178], which have previously been reviewed [179]. The most commonly utilised models are the $Cln3^{\Delta ex1-6}$ knockout mouse and the $Cln3^{\Delta ex7/8}$ knock-in mouse [176, 178]. Both lines reproduced many aspects of NCLs, such as locomotor abnormalities, memory/learning deficits, CNS storage material accumulation, gliosis and neurodegeneration [175, 178, 180-195]. These models provide useful tools for *in vivo* study of CLN3 functions and preclinical work of the CLN3 disease. However, although storage material accumulation is observed in the retina, retinal degeneration is found to be mild and late-onset in these mouse models [181, 186, 190, 192, 195], which is notable considering that visual loss and profound retinal degeneration is typically one of the earliest symptoms of CLN3 patients. However, retinal degeneration is remarkable when the CLN3 deficient mice are crossbred on a hypo-pigmented background [175, 196], which provides an option for utilising the mouse as a model for study of CLN3-related retinal abnormalities and corresponding preclinical work.

Recent studies employing CLN3 deficient mice showed that CLN3 deficiency led to pre-synaptic impairment, in several neuronal networks in the brain. Electrophysiological studies suggested pre-synaptic alteration in various brain regions

of CLN3 deficient mice, which preceded significant neuronal loss [182, 197, 198]. Widespread loss of synaptophysin (a pre-synaptic marker) was observed in the *Cln3* knockout mouse brain, and ultrastructural analysis revealed significantly decreased synaptic vesicles in the pre-synaptic terminals [197, 199]. Additionally, electrophysiological analysis revealed altered synaptic transmission and plasticity predominantly due to pre-synaptic changes [182, 197, 198]. Further study is still needed to address the association between synaptic abnormality, CLN3 disease progression and potential underlying molecular mechanisms.

Studies of the CLN3 deficient mouse models also suggested involvement of immune cells in CLN3 disease progression. . *In vitro* studies with primary microglial cultures from CLN3 deficient mice suggested CLN3 deficient microglia exhibited a more pro-inflammatory state upon neuronal lysate stimulation [200] and resulted in more neurotoxic effects on primary neurons [201]. Additionally, isolated peripheral antigen presenting cells (APCs) from the *Cln3* knockout mouse displayed dramatically increased levels of both total and membrane CD11c, which was not changed in other immune cells, indicating an APC-specific alteration [202]. Furthermore, treatment with immunomodulatory drugs reduced microgliosis and degeneration in the retina and the optic nerve of CLN3 deficient mice [196, 203]. Therefore, the immune response is probably not just a secondary response to other pathology, but likely contributes to the CLN3 disease progression and could be a potential therapeutic target.

As an attractive therapeutic strategy for NCLs, gene therapy has also been studied in CLN3 deficient mice. Intracranial administration of AAVrh10 vectors harbouring *CLN3* into newborn *Cln3*$^{\Delta ex7/8}$ mice produced improvements in neuronal lysosomal storage material accumulation and astrocytosis, but the effect on microgliosis or neurodegeneration was very limited at 16-18 months of age when transgene expression was still widely detected in the mouse brain [204]. Lack of microgliosis improvement may be caused by low microglial transduction. This study thus may provide additional evidence for the potential influences of microglial CLN3 deficiency. In another study, young *Cln3*$^{\Delta ex7/8}$ mice received intravenous injection of scAAV9 vectors with *CLN3* and displayed improvements of both motor abnormality and brain pathology including lysosomal storage material accumulation, astrocytosis and microgliosis at 5 months after treatment [205]. Particularly, the importance of promoter selection is highlighted in this study, as shown by significant improvement produced only in the mice with the viral vector employing the neuron-targeted promoter MeCP2 [205]. However, long-term therapeutic effects were not assessed in this study, which could be one of the reasons for the efficacy differences revealed by these two studies. In summary, although in one study neuronal CLN3 correction with gene transfer appears to be sufficient to provide phenotypic and pathologic improvements, long-term assessment after treatment is necessary for fully evaluating its therapeutic effects. Because CLN3 is a lysosomal membrane protein rather than secreted, CLN3 deficiency would only be rescued in the cells and regions transduced by viral vectors and no cross-correction would occur. In future, modifications of current gene transfer approaches may be needed for treating CLN3 disease.

Other therapeutic strategies have also been tested using the CLN3 mouse models. *Cln3* is expressed in brain endothelial cells [173] and blood-brain barrier damage is suggested in CLN3 disease [206, 207]. In particular, CLN3 deficiency is found to result in impaired membrane microdomain protein trafficking in CLN3-deficient brain endothelial cells, which provides more evidence and suggests a potential

mechanism for the blood-brain barrier damage [206]. Recently, the same research group employed carbenoxolone, a gap junction inhibitor, to investigate its effect on CLN3-associated endothelial cell deficits and brain pathology [169]. They showed that the endothelial cell deficit was rescued and the brain autofluorescence accumulation was largely reduced. Another approach aimed to restore cAMP levels, which are reduced in various brain regions of the *Cln3*$^{\Delta ex7/8}$ mouse [208]. Phosphodiesterase-4 inhibitors, inhibiting the degradative activity of Phosphodiesterase-4 on cAMP, were also tested for their therapeutic effects for CLN3 disease [208]. *Cln3*$^{\Delta ex7/8}$ mice receiving 6-month treatment showed restored cAMP levels in the brain and significant improvements in behavioural deficits and brain pathology such as lysosomal storage material accumulation and gliosis [208]. A study also suggested trehalose as a potential therapy for CLN3 disease [209]. They found that trehalose administration to the *Cln3*$^{\Delta ex7/8}$ mouse induced upregulation of autophagy/lysosomal genes by activating TFEB and improved brain pathology of the *Cln3*$^{\Delta ex7/8}$ mouse including autofluorescent storage material accumulation, gliosis and brain atrophy, and slightly increased the lifespan. These studies provide more potential options that could be considered as CLN3 disease therapies.

5.3 Zebrafish

The sole zebrafish homolog of human CLN3 is 446 amino acids long and has 48% identify at protein level (CLUSTAL W (1.81) multiple sequence alignment on Ensembl (www.ensembl.org)). No synteny is seen between the zebrafish Cln3 and human CLN3 loci. Expression of mRNA in the developing embryo has been described [210]. *cln3* mRNA is maternally deposited, zygotic expression is ubiquitous, but then becomes stronger in the brain.

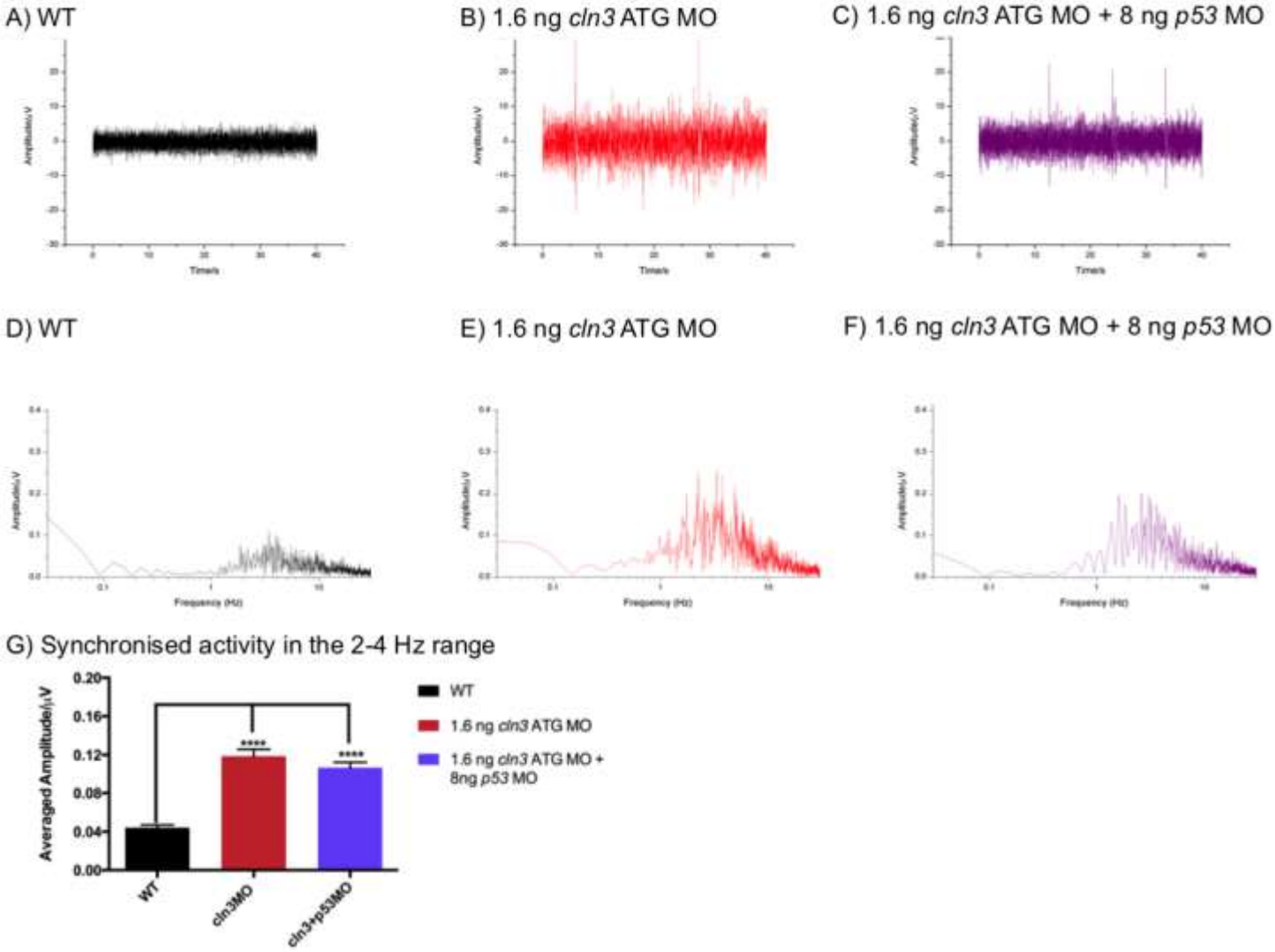
There is one strain available with a nonsense mutation in *cln3* (Table 3) but the phenotype has not yet been described. There were also three additional alleles reported at NCL2018, but these do not appear to have any phenotypes: they are adult viable and fertile, have normal locomotion and circadian rhythm, and normal electroretinograms [211]. It remains to be seen whether the Cln3 protein has been successfully knocked down.

Cln3 has been effectively knocked-down by antisense morpholino [210], demonstrating a phenotype similar to that seen in the zebrafish *tpp1*^{sa11} homozygotes and morphants. Phenotypes were obvious at 24 hpf, and by 4 dpf *cln3* morphants have a smaller retina, midbrain and hindbrain, the fourth ventricle is dilated, the heart is elongated and erythrocytes are not pigmented. Axon tracts are disrupted, GFAP is increased, proliferation is reduced and cell death increases. Furthermore, lysosomes are enlarged and subunit c of mitochondrial ATPase levels is increased within lysosomes. Mitochondria seem to have reduced membrane potential when assayed with Mitotracker Red. Activity is increased at 30 hpf, perhaps indicating hyperactivity, but the escape response and swimming ability is impaired by 4 dpf. It appears, through electroencephalography, that *cln3* morphants are experiencing seizures at 4 dpf and this correlates with the earlier hyperactivity. Epileptic zebrafish often travel further when seizing [212], but when a 4dpf *cln3* morphant is not able to swim, their seizures cannot be detected by the distance they travel.

On the surface, it seems that the lack of phenotype caused by mutation calls into doubt the specificity of the morphant phenotype. The lack of phenotype found by

others using morpholinos against *cln3* [213] also suggests we remain cautious when interpreting results.

Figure 2. Zebrafish injected with antisense morpholinos against cln3 exhibit seizures. Reproduced under the terms of the Creative Commons Attribution License from [210].



5.4 Fruit fly

The *Drosophila* Cln3 protein shares a predicted topology with the human protein and is 43% identical. Initial studies of *Cln3* used an overexpression strategy to generate gain-of-function phenotypes in the adult wing and eye that could be used for genome-wide screens to identify interaction pathways and genes [214]. Subsequently, a *Cln3* mutant was generated by the imprecise excision of a transposable element within the *Cln3* locus that deletes the N-terminal half of the locus, including the first third of the coding sequence [215].

Overexpression of *Cln3* in the developing wing led to a notching phenotype typical of classical mutations affecting Notch signalling and likely due to effects on vesicle trafficking. *Cln3* was also seen to interact strongly with the JNK pathway that is a central regulator of stress signalling. In the eye, overexpression of *Cln3* led to a glazing phenotype that could be blocked by increasing oxidative stress scavenging [214]. Taken together, the gain-of-function screens pointed to a role for Cln3 in the responses to stress signalling and this was confirmed by examining the loss-of-function *Cln3* mutant. *Cln3* mutant flies are hypersensitive to oxidative stress and have reduced lifespan, as are primary neurons cultured from *Cln3* deficient mice and CLN3 disease patient fibroblasts [215].

The neuromuscular junction (NMJ) of the late-stage *Drosophila* larva is a widely used model system to study the development and function of excitatory glutamatergic synapses and has provided insight into the neural functions of genes mutated in neurodegenerative disorders [216]. The NMJ is reduced in size in *Cln3* mutant larvae and when *Cln3* expression is knocked-down by RNAi expressed selectively in the neurons. Potentially, this phenotype is underpinned by a reduced activation of the JNK pathway in the mutants [128].

The endogenous Cln3 protein has proven difficult to detect in mammalian tissues due to low expression levels and non-specific antisera. This has left researchers reliant on a nuclear β-galactosidase reporter to detect where and when *Cln3* is expressed during development in the mouse [173] and overexpression of tagged forms of Cln3 have generally been used to study its sub-cellular localisation. Tuxworth and co-workers used recombineering to generate a *Drosophila* YFP-Cln3 knock-in that would be expressed at endogenous levels and under the control of the endogenous regulatory regions. Interestingly, this reporter demonstrated a low level and very restricted pattern of expression in the CNS. *Cln3* is expressed predominantly in glia that form the blood brain barrier and in these cells there is a prominent localisation of the Cln3 protein at the plasma membrane [217]. Single cell transcriptomics of the fly CNS support these findings and provides an explanation for the absence of autofluorescent storage material accumulating in neurons in *Cln3* mutant flies (Tuxworth, unpublished). However, *cln3* must also be expressed in glutamatergic neurons, including motor neurons – albeit at very low levels – since knockdown of expression specifically in these cells leads to synaptic phenotypes [128].

One organ where *cln3* is strongly expressed in the fly is the Malpighian tubule, the insect organ concerned with solute transport and water regulation. In this regard, the Malpighian tubule is orthologous to the mammalian kidney so it is probably not a coincidence that *Cln3* is also highly expressed in the mouse kidney inner medulla where it is required for transport of potassium into the urine [218]. Somewhat unexpectedly, the *Drosophila* knock-in revealed that the large majority of YFP-Cln3 protein was present at the apical domain in Malpighian tubules, abutting the lumen of the one cell-thick tubes. This was confirmed with a specific antibody capable of detecting the endogenous Cln3 protein [217]. While CLN3 is clearly lysosomal in cultured cells, potentially it has additional localisations in polarised epithelia.

5.5 Nematode

The *C. elegans* genome encodes three CLN3 homologues: *cln-3.1*, *cln-3.2* and *cln-3.3* [219, 220]. Deletion of *cln-3.1* resulted in a small but significant reduction in lifespan,

while *cln-3.2* deletion mutants exhibited a 6% decrease in the number of eggs laid per hermaphrodite [221], but otherwise the three single *cln-3* mutant worm strains appeared normal. In order to investigate whether the lack of strong phenotypes was due to redundancy, double and triple mutant strains were created [221]. Lifespan of the triple *cln-3* mutant was shorter than the single *cln-3.1* mutant, consistent with this idea; but the minor fecundity defect was no greater in the triple mutant than *cln-3.2* deletion mutants, suggesting that this phenotype is specific to *cln-3.2*. However, *cln-3* triple mutants exhibited normal movement and behaviour and no neuronal defects were observed. Similarly, there were no detectable changes in lipid accumulation or autofluorescence; and no signs of altered lysosome morphology or abundance when comparing *cln-3* triple mutant and wildtype worms [221]; although a recent study has reported a 10% increase in mitochondrial abundance in the terminal bulb of the pharynx [222]. To investigate the tissue specificity of *cln-3* expression in *C. elegans*, the putative promoters of each gene were fused to GFP and injected into worms. Based on the observed fluorescence signals, it can be concluded that *cln-3.1* expression is enriched in the intestine, *cln-3.2* in the hypodermis and *cln-3.3* in specific muscle cell types and the hypodermis [221]. The lack of observed GFP fluorescence in neuronal cells does not rule out the possibility that CLN-3 is expressed physiologically in neurons, but presumably this would be at relatively low levels compared to other tissues. Taken together, the lack of NCL-related phenotypes in the *cln-3* triple mutants and the lack of observable *cln-3* gene expression in neurons suggest that *C. elegans* is not the best organism for modelling CLN3 disease. Nevertheless, a recent study used the triple *cln-3* mutant strain to test for therapeutic effects of several different calcium channel blockers, observing that flunarizine could ameliorate the lifespan and mitochondrial phenotypes [222].

5.6 Social amoeba

The *Dictyostelium* homolog of human CLN3 is a 421 amino acid, 47 kDa transmembrane protein (Cln3) encoded by the *cln3* gene. Within a 429 amino acid region of similarity between Cln3 and CLN3, 27% of the amino acids are conserved and 45% are positive matches [62]. *cln3* is expressed at low levels during growth and the first 4 hours of development [83]. At the onset of aggregation, the expression of *cln3* increases dramatically reaching its highest level after 12 hours of development [83]. Expression then remains high during slug and fruiting body formation [83]. In growth and starved conditions, Cln3 localizes predominantly to the contractile vacuole (CV) system, and to a lesser extent, the Golgi complex and compartments of the endocytic pathway [78, 223, 224]. Loss of *cln3* has pleiotropic effects during the *Dictyostelium* life cycle. During growth, *cln3*-deficiency increases cell proliferation, alters cytokinesis, increases endo-lysosomal pH, and causes defects in osmoregulation [223, 225, 226]. During the early stages of multicellular development, *cln3*$^{-}$ cells accumulate autofluorescent storage material and display impaired adhesion (cell-substrate and cell-cell) and altered protein secretion, which delays aggregation [78, 224, 226]. Loss of *cln3* also alters nitric oxide homeostasis and causes cells to develop precociously [223, 226]. Finally, *cln3*-deficiency phenotypes in *Dictyostelium* can be suppressed by treating cells with the calcium chelator EGTA, which is consistent with aberrant calcium homeostasis reported in a mouse model of CLN3 disease [223, 224, 227]. Together, this work revealed previously undiscovered CLN3-dependent processes (e.g., protein secretion), as well as *cln3*-deficiency phenotypes that align with those observed in other genetic models of CLN3 disease (e.g., osmoregulation)

[147, 201, 206, 218, 228-232]. These data therefore provide strong evidence that the molecular function of CLN3 is conserved from *Dictyostelium* to human.

As discussed above, loss of *cln3* alters protein secretion in *Dictyostelium* [78]. During growth, *cln3*-deficiency affects the secretion and cleavage of proliferation repressors, which may help to explain the enhanced proliferation of *cln3*$^{-}$ cells [223]. When starved, loss of *cln3* alters the expression and secretion of cell adhesion proteins, which may contribute to the reduced adhesion and delayed aggregation of *cln3*$^{-}$ cells [224]. Based on these findings, mass spectrometry was used to further explore the role of Cln3 in protein secretion, which ultimately provided the first evidence in any system linking CLN3 to this conserved cellular process [78]. Twelve proteins that are not normally secreted by wild-type cells during starvation were detected in conditioned buffer from *cln3*$^{-}$ cells [78]. In addition, two proteins that play a role in regulating adhesion during the early stages of multicellular development were not detected in *cln3*$^{-}$ conditioned buffer, which may help to explain into the aberrant adhesion of *cln3*$^{-}$ cells [78, 224]. Label-free quantification revealed 42 proteins that were present in significantly higher amounts in conditioned buffer harvested from *cln3*$^{-}$ cells and three proteins that were present in significantly reduced amounts [78]. Bioinformatic analyses performed on these hits revealed an enrichment of proteins linked to vesicle-mediated transport, endocytosis, metabolism, and proteolysis; processes that have all been linked to CLN3 function in mammals [78, 206, 233, 234]. The proteomic analysis also revealed altered amounts of Tpp1F, Cln5, and CtsD in *cln3*$^{-}$ conditioned buffer, providing evidence that Cln3 modulates the secretion of NCL protein homologs in *Dictyostelium* [78]. A subsequent study used western blotting to confirm that *cln3*-deficiency increases the secretion of Cln5 [235]. That study also showed that Cln3 co-localizes with Cln5 at the CV system, which has been linked to unconventional protein secretion in *Dictyostelium* [235, 236]. The results of this work can now be used to fuel research in human systems to determine how altered protein secretion may play a role in the pathology underlying CLN3 disease. In fact, the role of CLN3 in protein secretion was explored in subsequent studies using mouse and human models. More specifically, glia and neurons isolated from *Cln3*-deficient mice were shown to display altered protein secretion profiles [201]. In addition, mass spectrometry was used to show that samples of brain and cerebrospinal fluid from the three major forms of NCL (CLN1, CLN2, and CLN3 disease) all have altered protein content compared to normal individuals [231]. Together, these studies, coupled with the proteomic analysis of conditioned buffer harvested from *Dictyostelium cln3*$^{-}$ cells, suggests that the role of CLN3 in protein secretion warrants further investigation.

RNA sequencing was performed to gain insight into the mechanisms underlying *cln3*-deficiency phenotypes during the early stages of multicellular development [226]. That study revealed 1153 genes that were differentially expressed in *cln3*$^{-}$ cells compared to wild-type cells during starvation [226]. STRING and GO term analyses showed an enrichment of differentially expressed genes linked to metabolic, biosynthetic, and catalytic processes [226]. In addition, these analyses showed that loss of *cln3* affects pathways related to Ras/MAPK signalling, glycan processing, and methionine and folate metabolism, which aligns with observations in mammalian models of CLN3 disease [237-242]. Among the differentially expressed genes were the *Dictyostelium* homologs of human *TPP1/CLN2* (*tpp1D* and *tpp1F*), *CTSD/CLN10* (*ctsD*), *PGRN/CLN11* (*grn*), and *CTSF/CLN13* [226]. Not surprisingly, *cln3*-deficiency was subsequently shown to alter Tpp1 and CtsD enzymatic activity [226].

These data provide further support for the molecular networking of NCL proteins. Finally, loss of *cln3* alters the expression and activity of lysosomal enzymes during starvation, specifically N-acetylglucosaminidase, alpha-mannosidase, and beta-glucosidase [226]. These findings are consistent with the altered activity of these enzymes in post-mortem gray matter from NCL patient brains [243].

RNA sequencing was also used to better understand the role of Cln3 in osmoregulation in *Dictyostelium* [225]. During hypotonic stress, 320 genes were differentially expressed in *cln3*$^{-}$ cells compared to wild-type cells, and 162 genes were differentially expressed during hypertonic stress. Bioinformatic analyses showed that the differentially expressed genes are linked to developmental processes, which was confirmed by work showing that *cln3*$^{-}$ cells develop abnormally when subjected to osmotic stress [225]. Genes differentially expressed during hypotonic stress are also linked to metabolic processes [225]. Genes involved in transport and catalysis were differentially expressed during both osmotic stress conditions, which supports the role of Cln3 in protein secretion, including the secretion of proteases [78, 225]. Finally, loss of *cln3* increases the expression of *tpp1A* during hypertonic stress, which was shown to correlate with increased Tpp1 activity [225]. In total, this work supports a role for Cln3 in osmoregulation, which should fuel research in mammalian models of CLN3 disease to determine how altered osmoregulation contributes to clinical phenotypes observed in NCL patients. This is especially relevant considering that osmoregulatory defects have also been reported in mammalian models of CLN3 disease [218, 229].

6. CLN4 disease

Homozygous mutations in the transmembrane protein DnaJ homolog subfamily C member 5 (DNAJC5), also known as cysteine string protein alpha (CSPa), cause CLN4 disease.

6.1 Large animals

No large animal models for CLN4 disease have been identified.

6.2 Rodents

The mouse homolog of CSP, a 198 amino acid protein encoded by mouse *Dnacj5* gene, shares 99% identity with human CSP (CLUSTALW (2.1) Multiple Sequence Alignment on Ensembl (www.ensembl.org)). CSP is a well-known synaptic vesicle protein. Before human *DNAJC5* mutations were identified to cause CLN4 disease, *Dnajc5* knockout mice had been produced to study CSP functions in synapses and neurodegenerative diseases. The *Dnajc5* knockout mouse develops progressive synaptic- and neuro-degeneration, retinal degeneration, sensorimotor dysfunctions and a severely shortened lifespan [244, 245]. Further studies show that loss of the SNARE protein SNAP-25 in presynaptic compartments is the predominant cause of CSP-deficiency-associated neurodegeneration [246, 247]. Overexpression of human alpha-synuclein, which also performs a SNARE chaperoning function, in the CSP-deficient mouse rescues the neuropathology in the brain, rescues the motor impairments, and improves mouse survival [244]. More research into CLN4 disease is obviously still needed and the *Dnajc5* knockout mouse could be a valuable

mammalian model for investigating CLN4 disease mechanisms and developing therapies.

6.3 Zebrafish

There are two versions of DNAJC5 in zebrafish, called Dnajc5aa and DNAJC5ab. Dnajc5aa is on chromosome 8 in a region with no synteny with the human homolog. This protein is 202 amino acids and has 81% identity at the protein level (CLUSTAL W (1.81) multiple sequence alignment on Ensembl (www.ensembl.org)). Dnajc5ab, on the other hand, is on chromosome 23 in a region with a small amount of synteny with the human homolog, encompassing human DNAJC5, GUD8, SLC17A9, BHLHE23 on chromosome 20. Zebrafish Dnajc5ab is 199 amino acids long and has 75% identity at the protein level (CLUSTAL W (1.81) multiple sequence alignment on Ensembl (www.ensembl.org)). Hence, synteny suggests that Dnajc5ab is the true homolog, but identity suggests that Dnajc5aa is more likely to have homologous function, though there may not be a large difference in function as the two homologs are 82% identical at the protein level. Both zebrafish proteins were found in synaptosomes from central synapses, similar to mouse Dnajc5 which is also found at a higher level in synaptosomes compared to post-synaptic densities [121]. Clearly more investigation is needed and it is likely that both genes will need to be mutated to make a zebrafish model of CLN4 disease. To date, no one has yet performed any experiments with these zebrafish genes.

6.4 Fruit fly

Cysteine string protein has perhaps been better studied in *Drosophila* than in any other model system since it was first identified in 1990 as the antigen recognised by one of a set of classic neurobiology monoclonal antibodies generated from *Drosophila* heads [248]. It was subsequently named for its long string of cysteines. The single *Csp* gene in flies is widely expressed, including in all neurons. The protein localises to the neuropil of the larval and adult brain and is found in the synaptic terminals of the larval neuromuscular junction but not in the cell bodies of neurons. Mutations in *Drosophila Csp* severely affect neural transmission leading to locomotor defects, spasms and paralysis and a progressive neurodegeneration due to synapse failure [249, 250]. The phenotypes are somewhat temperature sensitive, consistent with a role for *Csp* as a chaperone. *Csp* appears to play several different roles in synaptic transmission. These include Ca^{2+}-triggered exocytosis, regulation of pre-synaptic Ca^{2+} levels and protection from thermal stress. Endocytosis of the synapse appears to be intact, however, in *Csp* mutants and not all of Csp's pre-synaptic functions require binding to its chaperone partner, Hsc70 [251, 252].

6.5 Nematode

C. elegans has a single homologue of the human *DNAJC5* gene mutated in CLN4 disease: *dnj-14*. The worm DNJ-14 protein displays extensive similarity to the human *DNAJC5* gene product, cysteine string protein (CSP), in the DnaJ domain and the central cysteine string domain, with more sequence divergence apparent in the N- and C-terminal regions [253]. Notably, the two leucine residues within the cysteine string domain that are mutated in CLN4 disease (L115/L116) are conserved in *C. elegans*

DNJ-14 (L138/L139). To date, there has been no report of a direct CLN4 model whereby these leucines have been specifically mutated to mirror the human disease. However, because CLN4 disease ultimately results in the loss of CSP from its physiological location on synaptic vesicles due to aggregation and retention in the neuronal cell body [254-256], null mutants of *C. elegans dnj-14* can be used as surrogate models of CLN4. The *ok237* deletion allele contains a 2229 bp deletion that eliminates the great majority of the *dnj-14* open reading frame and its promoter, and so is a molecular null exhibiting a complete loss of DNJ-14 protein expression. Young *dnj-14* (*ok237)* mutant worms are essentially wild type for most phenotypes, although a very small reduction in locomotion is apparent. As the worms age, this locomotor impairment becomes progressively worse and a severe age-dependent chemosensory defect becomes apparent [253]. Normally, worms sense and move towards attractive sources, such as food and certain volatile compounds, but by 6-days-old, *dnj-14* (*ok237)* mutant worms have lost this chemotactic response. At 9-days-old, degeneration of sensory neurons in the head of *dnj-14* (*ok237)* mutant worms becomes apparent, as seen by loss of fluorescence from GFP-labelled neurons, suggesting that the functional chemosensory impairment precedes morphological neurodegeneration. In keeping with these progressive, age-dependent phenotypes, *dnj-14* (*ok237)* mutant worms exhibit a considerably reduced lifespan [253]. The large deletion in *dnj-14* due to the *ok237* allele also extends into a neighbouring gene, *glit-1*, raising the potential concern that the observed phenotypes could be due to effects on *glit-1*. However, the lifespan and behavioural phenotypes of the *ok237* allele were rescued by transgenic wild type *dnj-14*, and similar effects on lifespan and behaviour were observed using a smaller *dnj-14*-specific deletion allele, *tm3223*, or using RNAi knockdown of *dnj-14* in an RNAi hypersensitive strain [253]. Taken together, these data indicate that the *C. elegans dnj-14* null mutant model of CLN4 mirrors the human disease in terms of its late onset, progressive effects on the sensorimotor system, neurodegeneration and premature death.

Genome-wide transcriptional profiling via DNA microarrays revealed a reduction in expression of genes involved in the ubiquitin proteasome system (UPS) – especially E3 ubiquitin ligases - in both the *ok237* and *tm3223* mutant strains [257]. Consistent with this, use of a GFP-tagged ubiquitin reporter revealed a decrease in ubiquitylated protein degradation in *dnj-14* mutants [257]. These results indicate that disruption of the synaptic vesicle chaperone *dnj-14* leads to reduced expression of UPS-related proteins, which in turn impairs proteasome-mediated protein degradation in the *C. elegans* model of CLN4. Given that proteasome inhibitor drugs are therapeutic in *dnajc5* knockout mice [258], there appears to be an evolutionarily conserved role for the UPS in CLN4 disease The specific over-representation of E3 ubiquitin ligase components observed in worms [257] suggests that proteins and complexes upstream of the proteasome may potentially be beneficial therapeutic targets worthy of exploration in more complex models.

One advantage of *C. elegans* over mammalian models is that compound screening can be performed quickly and cheaply with no ethical restraints. A focused drug screen identified the polyphenolic compound, resveratrol, as being able to ameliorate the various phenotypes of the *C. elegans dnj-14* mutants [253]. Resveratrol's neuroprotective effects are often ascribed to its ability to activate the protein deacetylase, SIRT1, but its therapeutic action in the *dnj-14* model is unaffected by deletion of the worm SIRT1 orthologue, *sir-2.1*. Instead, it appears that resveratrol acts by inhibiting phosphodiesterases and thereby increasing cyclic AMP levels, as its

effects on the *dnj-14* model are mimicked by the phosphodiesterase inhibitor rolipram [253]. Another compound that was found to rescue the *dnj-14* mutant is the anti-epileptic drug, ethosuximide [259]. Ethosuximide was shown to act via the DAF-16/FOXO transcription factor, which is an evolutionarily conserved master regulator of stress resistance and longevity. Indeed, ethosuximide is effective in cell culture models of Huntington's disease [259] and in a rat *in vivo* acute Alzheimer's disease model [260], suggesting that it has general neuroprotective properties that could extend to other NCLs. Most recently, a screen of ethosuximide-related compounds identified α-methyl α-phenylsuccinimide to be over 100 times more potent than ethosuximide in a *C. elegans* TDP-43 neurodegeneration model [261]. It will be interesting to test whether the various neuroprotective compounds identified using the *C. elegans dnj-14* model also have therapeutic activity in mammalian NCL models and hence have translational potential.

6.6 Social amoeba

The *Dictyostelium* homolog of human DNAJC5 appears to be the protein product of an uncharacterized gene, *DDB_G0290017*, which encodes a 176 amino acid, 20 kDa protein (DDB0306688). Within an 84 amino acid region of similarity between DDB0306688 and DNAJC5, 44% of the amino acids are conserved and 64% are positive matches. The expression of *DDB_G0290017* is highest during growth and the protein product of the gene localizes to the macropinocytic pathway [80, 83]. Upon starvation, the expression of *DDB_G0290017* decreases dramatically, reaching its lowest level as cells are aggregating into multicellular mounds [83]. Expression then remains low during the mid-to-late stages of development [83]. This expression profile suggests that the protein may function primarily during the growth phase of the *Dictyostelium* life cycle. As previously reported, the *Dictyostelium* genome encodes another protein (Ddj1, *Dictyostelium* DnaJ homolog 1) that shares sequence similarity with human DNAJC5 [62]. Ddj1 localizes to the centrosome, phagosome, and macropinocytic pathway and is predicted to play a role in phagocytosis [80, 262, 263]. However, Ddj1 is significantly larger (411 amino acids, 46 kDa) than the 198 amino acid human DNAJC5 protein and the alignment between the two proteins only encompasses a 69 amino acid region of similarity [62]. For these reasons, it is more likely that the protein product of the uncharacterized gene *DDB_G0290017* is the *Dictyostelium* homolog of human DNAJC5. Knockout mutants of *DDB_G0290017* and *ddj1* have not yet been generated. Therefore, future research will be required to determine which of the above-described proteins is the true homolog of human DNAJC5 in *Dictyostelium*.

7. CLN5 disease

Homozygous mutations in the lysosomal protein CLN5 cause CLN5 disease.

7.1 Large animals

7.1.1. CLN5 disease in dogs

CLN5 mutations have been described in several breeds of dog. The Border collie NCL mutation (*CLN5:c.619C > T* ; [264]) is well known in breeding circles and pedigree dogs are routinely screened. More recently, the same CLN5 mutation was

identified in Australian cattle dogs [265]. In addition, a different mutation (*c.934_935delAG*) causes CLN5 disease in Golden Retrievers [266]. Border Collies and Golden Retrievers with CLN5 disease display indistinguishable clinical development with symptom onset at around 13 months of age with terminal disease by three years old. These dogs may provide a useful addition/alternative to the sheep model for preclinical studies.

7.1.2 CLN5 disease in Borderdale sheep

The Borderdale sheep NCL was first described by Jolly et al. in 2002 [267]and subsequently confirmed to be caused by a mutation in CLN5 by Frugier and colleagues [268].

Gene therapy efforts in the sheep flocks were initiated in 2008 with testing of lentiviral vectors [269, 270] and subsequently AAV9 vectors [270]. Initial testing using vectors expressing GFP, showed that lentiviral- mediated gene delivery was limited to the site of parenchymal injection or, following ICV injection, the ependyma and sub-ependymal layers. This low-level transduction was however sufficient for suppressing disease progression in CLN5 disease. AAV9 resulted in widespread expression from single sites of injection, however inflammatory responses were seen after parenchymal injections of AAV9-GFP and current studies focus on ICV administration ([270] and in preparation). Both lentivirus and AAV9 vectors expressing CLN5 protected sheep from development of behavioural deficits and pathology [271] when administered pre-symptomatically and AAV9-*CLN5* stabilised disease when given after disease onset [272]. AAV serotypes differ in tropism between species and to date only AAV7 and 9 have been tested in sheep models [271]. It will be important when heading toward clinical trials to optimise the vector serotype used and test safety and toxicity in NHPs. These preclinical studies in sheep required the development of biomarkers. Extensive work has resulted in a suite of behavioural and clinical measurements [270], and electroencephalography (EEG), computed tomography (CT) [273], ERG [274] and imaging biomarkers that can be used to test additional therapeutic strategies. Combining electromyography (EMG), electrooculography (EOG) and electroencephalography (EEG) measurements allows sleep status to be evaluated [275, 276]. These later studies identified abnormal epileptiform activity and sleep abnormalities in CLN5 mutant sheep that could be traced longitudinally, similar to that seen in CLN5 patients. Combining these biomarkers will enhance the ability to determine efficacy in therapeutic trials in future.

7.2 Rodents

The mouse homolog of CLN5, a 341 amino acid protein encoded by mouse *Cln5* gene, shares 74% identity with human CLN5 (CLUSTALW (2.1) Multiple Sequence Alignment on Ensembl (www.ensembl.org)). *Cln5* is widely expressed throughout the mouse brain and upregulation during brain development is remarkably observed [277, 278]. *In situ* hybridization and immunohistochemistry on postnatal brain tissue reveal prominent *Cln5* expression in cerebellar Purkinje cells, cerebral cortical neurons, hippocampal and hypothalamic neurons [277], though a different study on primary cell cultures suggests higher expression levels in glial cells than in neurons [278].

A *Cln5* knockout mouse line has been developed by disrupting exon 3 of *Cln5* gene to model CLN5 disease [279]. Initial characterization of the model shows that it

reproduces several aspects of CLN5 disease such as progressive visual loss beginning from 13 weeks of age and autofluorescent storage material accumulation in the brain and retina [279]. Ultrastructural analysis of the cortical neurons with storage material accumulation shows typical fingerprint lamellar profiles [279]. Loss of GABAergic interneurons is observed in various brain regions by 6 months of age, while no obvious brain atrophy occurs at this stage [279]. A later study has further characterized this model, showing that significant neurodegeneration occurs as early as 4 months in the cerebral cortex and later in the thalamus while obvious brain atrophy and thinner cortical layers are observed by 12 months of age [280]. Also, recent research points to other neurobiological changes that might contribute to CLN5-deficiency-related neurodegeneration. Neurogenesis is found to be impaired in the *Cln5* knockout mouse brain, as shown by impaired generation of mature neurons with normal neuritic morphology, in spite of increased neural progenitor cell proliferation [281]. Interestingly, astrocytosis appears to occur much earlier (1 month of age) compared to neurodegeneration [280], suggesting potential direct contribution of CLN5-deficient astrocytes to the disease and/or perhaps any early neural functional changes that could induce astrocytosis prior to neuronal loss. More research into the *Cln5* knockout mouse at the stages prior to significant neurodegeneration also suggests early microgliosis and myelination deficits in the brain, plus systemic serum cholesterol elevation [278], which provides clues for potential unknown functions of CLN5 relevant to CLN5 disease, particularly in cell types other than neurons. Additionally, since progressive visual loss is a typical and early manifestation of CLN5 disease and the *Cln5* knockout mouse also shows such a phenotype, the retinal pathology is of great interest in CLN5 studies.

Characterization of retinal pathology in the *Cln5* knockout mouse reveals progressive retinal photoreceptor cell apoptosis and glial infiltration beginning by 1 month of age [282]. The retinal outer nuclear layer shows dramatic atrophy by 3 months of age and further progresses with ageing [282]. Electroretinogram shows early-occurring and progressive rod-mediated functional decline, and cone-mediated functional decline at late stages [282]. In summary, the *Cln5* mouse model represents an invaluable *in vivo* tool for studying CLN5 functions and CLN5 disease. However, further investigation of the model is still needed, such as to reveal deficits in behavioural performance and neurobiological changes relevant to the disease, as well as the mouse survival and endpoint manifestations, which could be particularly important for employing this model in novel therapy development and assessment.

7.3 Zebrafish

The zebrafish has one homolog of CLN5, found on chromosome 9 and encoding a protein of 329 amino acids. Zebrafish Cln5 is 55% identical to the human protein (CLUSTAL W (1.81) multiple sequence alignment on Ensembl (www.ensembl.org)) and there is a small syntenic region encompassing CLN5, FBXL3, MYCBP2 and SLAIN1. No experiments appear to have been performed to investigate zebrafish Cln5.

7.4 Fruit fly

Drosophila has no clear homologue of the *CLN5* gene.

7.5 Nematode

C. elegans does not have a clear homologue of the *CLN5* gene.

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7.6 Social amoeba

The *Dictyostelium* homolog of human CLN5 is a 322 amino acid, 37 kDa protein (Cln5) encoded by the *cln5* gene [62]. Within a 301 amino acid region of similarity between Cln5 and CLN5, 30% of the amino acids are conserved and 47% are positive matches [62]. The *Dictyostelium* genome also encodes three additional uncharacterized proteins that share sequence similarity with human CLN5 (DDB0346861, DDB0308005, and DDB0346698) [283]. However, the genes encoding these proteins (*DDB_G0271546*, *DDB_G0279757*, and *DDB_G0293236*) are all expressed at significantly lower amounts compared to *cln5* [83]. The most significant discovery from work in *Dictyostelium* was that both *Dictyostelium* Cln5 and human CLN5 have glycoside hydrolase activity [283]. This knowledge can now be used to fuel research in human models to develop therapies to restore the potential loss of glycoside hydrolase activity in patient cells. As in human cells, loss of Cln5 function in *Dictyostelium* causes an accumulation of autofluorescent storage bodies within cells [235]. Recent work has shown that Cln5 is glycosylated in the ER and then trafficked to the cell cortex where it appears to be secreted via the CV system [78, 235, 283]. A proteomics-based analysis has also localized Cln5 to the macropinocytic pathway [80]. The secretion of Cln5 in *Dictyostelium* is consistent with the presence of a signal peptide for secretion in human CLN5, as well as the detection of CLN5 in conditioned media from cultured mammalian cells [283-285]. When *Dictyostelium* cells are starved, autophagy is induced, providing nutrients for multicellular development [286]. Intriguingly, Cln5 secretion is reduced when wild-type cells are treated with lysosomotropic agents, suggesting that autophagic mechanisms regulate the secretion of the protein [235]. This result is in line with observations from mouse and human cell models of CLN5 disease that link the function of CLN5 to autophagy [282, 287]. The expression of *cln5* increases dramatically during the early stages of development [83]. Upon starvation, *cln5*$^{-}$ cells display reduced cell-substrate adhesion, which is exacerbated when cells are treated with lysosomotropic agents, further supporting a link between Cln5 function and autophagy [235]. During this stage of the life cycle, *cln5*$^{-}$ cells also display reduced cell-cell adhesion [235]. These findings are consistent with the reduced adhesion of cells derived from *Cln5*-deficient mice and CLN5 disease patients [288, 289]. As a likely consequence of the reduced adhesion, *Dictyostelium cln5*$^{-}$ cells also display defects in chemotaxis during the early stages of multicellular development [235]. Finally, *Dictyostelium* Cln5 interacts with lysosomal enzymes (e.g., beta-glucosidase, alpha-mannosidase), cysteine proteases, other homologs of NCL proteins (e.g., Tpp1B, CtsD, DDB0252831/similar to CTSF), and proteins linked to Cln3 function in *Dictyostelium* (e.g., AprA, CfaD, CadA) [283]. In addition, ten Cln5-interactors are differentially secreted by *cln3*$^{-}$ cells, providing further support for the molecular networking of NCL proteins in *Dictyostelium* [78, 283]. In total, work from *Dictyostelium* shows that Cln5 is secreted during the early stages of multicellular development and suggests that it may function as a glycoside hydrolase to modulate adhesion. Future studies can now utilize this knowledge to examine the role of extracellular CLN5 in the pathology underlying NCL.

8. CLN6 disease

Homozygous mutations in the CLN6 transmembrane protein cause CLN6 disease.

8.1 Large animals

8.1.1 CLN6 disease in sheep

The CLN6 South Hampshire (New Zealand) flock has been key to understanding pathology, and more recently, testing therapies in a large animal brain. *In vivo* pathology and identification of storage has been extensively reviewed previously [290]. Here we focus on data published since 2013 and reported at NCL2018 (www.ncllondon2018.com). The availability of tissue samples continues to allow comparative pathology and testing of biomarkers. Embryonic sheep brain preparations, first developed in the 1990s [291, 292] have been recently used to establish early (pre-storage body) changes in the autophagy/lysosome and endocytotic pathways [293], and for testing of drug [293, 294] and gene therapy strategies [291] prior to *in vivo* testing. The relatively large brain provides ample cells for multiple studies and refinement of methods for storage of single cell suspensions in liquid nitrogen provides for a continued supply of cells from the same group of animals [269]. Similarly, use of body fluids, including urine, have been used to establish additional biomarkers that might be useful in preclinical trials [295] . Drug trials in sheep are limited by costs (mg/kg required compared to small models) and metabolic differences between ruminants and humans. Based on earlier reports that glial inflammation precedes neuronal loss in CLN6 sheep [296], Kay and Palmer tested chronic oral administration of minocycline, an antibiotic with anti-neuroinflammatory effects. While pharmacologically relevant levels of minocycline were detected in the brain, no significant effects on neuroinflammation or disease progression were found [297].

The South Hampshire [12] and Merino [298] flocks have different CLN6 mutations, The South Hampshire sheep have a deletion in exon 1. The Merino sheep have a point mutation c.184C>T resulting in p.Arg62Cys which is the subject of proteasome mediated degradation (Neverman and Hughes, unpublished). While mortality is reportedly earlier in the Merino (19-27 months compared to 25-30 months in South Hampshire) this may be reflective of pastural conditions between sites rather than disease associated differences. The Merinos have not yet been used in preclinical studies, however preparatory biomarker and clinical course studies have been done on a small number of sheep [299]. More extensive analysis and experience with the South Hampshire flock over the last 3 decades have established a suite of pathological and behavioural data sets (reviewed in [290]). Most recently this flock have been used for gene therapy trials [290]. The only effective gene therapy in CLN6 mutant sheep was in pre-symptomatic sheep treated with AAV9. This was effective in 1/3 and 1/6 sheep [272] in respective trials suggestive that gene therapy is possible in this form of the disease. Establishing why only some sheep respond to treatment will be important going forward with these preclinical studies.

8.2 Rodents

The mouse homolog of CLN6 shares 92% amino acid identity with human CLN6 (CLUSTALW (2.1) Multiple Sequence Alignment on Ensembl (www.ensembl.org)). The mouse *Cln6* gene is mapped to chromosome 9. Upregulation of *Cln6* expression during postnatal development is observed in the developing mouse brain, especially in the cerebral cortex and cerebellum [300]. In the adult mouse, *Cln6* is widely expressed throughout the brain, and *in situ* hybridization reveals localized expression in the cerebral cortex, cerebellar Purkinje cell layer, and hippocampal DG and CA1 cell body layers [300]. An insertion mutation in exon 4 of mouse *Cln6* resulting in a frameshift truncation is the cause of a murine form of NCL (*nclf*), thus the *nclf* mouse appears to be a naturally occurring mouse model for human CLN6 disease [19-21].

Behavioural manifestations and CNS pathology of the *nclf* mouse have been characterized. Although the mice appear to be normal until 8 months of age when they start to develop progressive hind limb paralysis [19], research employing rotarod tests reveals that significantly impaired motor coordination is evident by 3 months of age [301]. Impaired memory and learning performance is also reported in the mouse [301]. Characterization of the pathology in the *nclf* mouse shows accumulation of lysosomal storage material in the CNS beginning postnatally [19], and by 12 months of age strong autofluorescence accumulation is widely observed throughout the brain [302]. Early-onset gliosis in the *nclf* mouse brain is another hallmark of the model, which is consistent with the CLN6 sheep model [296, 302]. Furthermore, prior to observation of neurodegeneration, the *nclf* mouse displays significant synaptic and axonal pathology at the pre/early-symptomatic stages, as shown by cortical spine density decrease, dysregulated synaptic protein levels in the thalamocortical system, demyelination and axonal degeneration in the spinal cord and decreased internal capsule volume [19, 301, 303]. Such pathology suggests the existence of early neural functional changes potentially contributing to the behavioural phenotype of the model before neuronal loss. Additionally, progressive retinal degeneration is another early event in the *nclf* mouse [301, 304, 305]. Similar to the brain pathology, astrocytosis and autofluorescent storage material accumulation are also observed in the retina at early stages [304, 305]. The mouse then exhibits impaired visual acuity by 8 months of age [304, 305]. Interestingly, a recent study has found a significant difference in the disease progression between male and female *nclf* mice, with the female mouse showing accelerated autofluorescence accumulation and behavioural deficits [306].

As CLN6 disease is a monogenetic disease due to CLN6 deficiency, gene therapy is an attractive approach. An earlier study testing effects of AAV gene therapy on the retinal phenotype of the *nclf* mouse showed that intravitreal administration of AAV variant 7m8 vectors with *CLN6* rescued the photoreceptor cell loss in the retina [307]. A preclinical study of AAV9 gene therapy, where the human CLN6 gene was under the control of the chicken β-actin promoter, using the *nclf* mouse was recently reported at NCL2018: significant improvements were seen in motor performance, learning and memory and survival, as well as a reduction in autofluorescent storage material [308]. Subsequent test on Cynomolgus Macaques demonstrated no adverse effects and high levels of the transgene in the brain and spinal cord [308]. Safety trials for intrathecal gene therapy for CLN6 disease have since begun [309].

8.3 Zebrafish

Zebrafish have two homologues of CLN6, called Cln6a and Cln6b. Cln6a is a 298 amino acid protein on chromosome 7 with 68% identity with human CLN6

(CLUSTAL W (1.81) multiple sequence alignment on Ensembl (www.ensembl.org)). Only two genes, CLN6 and CALML4, are syntenic. Cln6b may code for three proteins of different lengths – 309, 310, 241 amino acids. It is on chromosome 26, has synteny over four genes, CALML4, CLN6, FEM1B, ITGA11, but has only 57% identity (for the 310 amino acid protein) (CLUSTAL W (1.81) multiple sequence alignment on Ensembl (www.ensembl.org)).

Three alleles of *cln6a* have been generated (Table 3). A further four alleles were reported at NCL2018. One has been examined for a phenotype and homozygotes appear to be normal, adult viable and fertile, with normal electroretinograms [211]. Knock down of the Cln6a protein has not yet been demonstrated in these alleles.

8.4 Fruit fly

Drosophila has no clear homologue of the *CLN6* gene.

8.5 Nematode

C. elegans does not have a clear homologue of the *CLN6* gene.

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8.6 Social amoeba

The *Dictyostelium* genome does not encode a homolog of human CLN6.

9. CLN7 disease

CLN7 disease is caused by homozygous mutations in the transmembrane protein called Major facilitator superfamily domain-containing protein 8 (MFSD8).

9.1 Large animals

9.1.1 CLN7 disease in dogs

Dog models of CLN7 NCL have recently been described in the Chinese -crested dog [310] and Chihuahua [311] (Table 1), however research colonies are not currently established.

9.1.2 CLN7 disease in monkeys

The first genetically confirmed monkey NCL was reported in 2018 [18]. The Japanese macaque disease is caused by a frameshift mutation in *CLN7*, *c769delA*, which causes a truncated protein, MFSD8. Monkeys presented clinically at around 5 years with symptoms including incoordination, ataxia, hindlimb weakness and impaired balance. Six monkeys were followed from three until six years. Progressive loss of cerebral and cerebellar volume was observed by magnetic resonance imaging (MRI), and retinal degeneration tested using optical computed tomography (OCT) and ERG. Hallmark pathology including autofluorescent storage material and gliosis were also found in the brain at necropsy. This model is now being established as a research

colony at the Oregon National Primate Research Center, providing the first NHP model with which to study therapeutic strategies.

9.2 Rodents

The mouse homolog of MFSD8 shares 81% amino acid identity with human MFSD8 (CLUSTALW (2.1) Multiple Sequence Alignment on Ensembl (www.ensembl.org)). The first *Mfsd8* deficient mouse model was generated in 2014, reproducing typical NCL-like pathology such as autofluorescent storage material accumulation and gliosis throughout the brain and retinal degeneration [312]. Since this mouse model still expressed low levels of *Mfsd8*, the same research group developed another *Mfsd8* knockout mouse model with no detected *Mfsd8* expression in the brain [313]. The knockout mouse exhibited clasping, hind limb paralysis, seizures and a shortened lifespan [313]. Pathology in the brain included autofluorescent storage material accumulation, accumulated mitochondrial ATP synthase subunit c and saposin D, and brain gliosis [313]. Furthermore, upregulated expression of lysosomal cathepsins and accumulated autophagic proteins in the brain of the knockout mouse suggested impaired autophagy-lysosomal pathway [313]. Additionally, the knockout mouse also showed retinal pathology including retinal degeneration, microgliosis and lysosomal dysfunction [314]. In future, this mouse model, together with the monkey CLN7 disease model [18], could be a valuable tool for pre-clinically studying novel therapies for CLN7 disease.

9.3 Zebrafish

Zebrafish chromosome 12 harbours the homolog of human MFSD8 with a syntenic region that spans MFSD8, ZCCHC4, ABLIM2 and AFAP1. Zebrafish Mfsd8 has 504 amino acids with 61% identity with human MFSD8 protein (CLUSTAL W (1.81) multiple sequence alignment on Ensembl (www.ensembl.org)). Five mutations exist in the gene. They have not yet been investigated to see if they provide a suitable model for CLN7 disease (Table 3). Three further alleles were created by gene editing and one was examined for phenotypes in homozygotes but they were adult viable and fertile with no obvious health defects, as presented at NCL2018 [211]. The consequence of these mutations on the protein still needs to be examined.

9.4 Fruit fly

Drosophila has one MFSD8/CLN7 homologue. A recent study generated a YFP-Cln7 fusion knock-in construct by CRISPR/Cas9 gene editing to allow the expression and sub-cellular localisation of Cln7 protein to be assessed without overexpression. This line, together with a second line in which the *Cln7* promoter drives expression of a nuclear RedStinger protein, reveals widespread expression of *Cln7* but a surprisingly restricted expression within the CNS. *Drosophila Cln7* is largely restricted to glia in the larval CNS and only expressed in neurons in the developing visual system [217]. As with Cln3, Cln7 is expressed in the glia that comprise the blood brain barrier along with other glia along the midline of the ventral nerve cord. At the larval neuromuscular junction, Cln7 expression is restricted to the post-synaptic muscle and apparently absent from the pre-synaptic nerve. Interestingly, Cln7 is recruited to the post-synaptic membrane where it co-localises with Dlg, the *Drosophila* homologue of

PSD-95. This mirrors the localisation of human Cln7 in receptor the outer plexiform layer of the retina where it also co-localises with PSD-95 [315]. A *Cln7* mutant generated by imprecise excision of a transposable element reveals a requirement for *Cln7* for the normal development of the synapse (Connolly et al, submitted: available at bioRxiv: https://www.biorxiv.org/content/10.1101/278895v3). Given the localisation of Cln7 to the post-synaptic side of the synapse, this suggests a role in retrograde signalling across the synapse.

9.5 Nematode

The *C. elegans* genome encodes a large number of proteins with significant similarity to the human MFSD8 protein based on BLASTP analysis. Of these, six display more than 29% amino acid identity over 95% of the full length protein sequence: Y53G8AR.7, ZK550.2, F14D7.6, T28C12.1, T28C12.2 and W05E10.1 (Table 4). All that is known about the function of these genes is that RNAi knockdown of Y53G8AR.7 increases resistance to the oxidative stressor, paraquat, and increases lifespan [316]. It is unclear which of the worm MFSD8 homologues corresponds to the human protein and it may be that there is considerable genetic redundancy among the numerous paralogues. If so, construction of strains containing multiple mutation might be required. Given that there are no currently available deletion mutants, this would represent a significant challenge. It therefore seems unlikely that a worm model of CLN7 disease will be developed in the near future.

9.6 Social amoeba

The *Dictyostelium* homolog of human MFSD8 is a 498 amino acid, 55 kDa protein (Mfsd8) encoded by the *mfsd8* gene [62]. Within a 530 amino acid region of similarity between Mfsd8 and MFSD8, 29% of the amino acids are conserved and 47% are positive matches [62]. Like other homologs of NCL proteins in *Dictyostelium*, Mfsd8 localizes to the macropinocytic pathway [80]. The expression of *mfsd8* remains relatively constant throughout the *Dictyostelium* life cycle, but peaks after 8 hours of development, suggesting that the protein may play a role in cAMP-mediated chemotaxis and aggregation [83]. Future work that generates and characterizes a *Dictyostelium mfsd8* knockout mutant is likely to provide novel insight into the precise function of MFSD8 in human cells and the processes it regulates.

10. CLN8 disease

CLN8 disease is caused by homozygous mutations in the transmembrane protein CLN8.

10.1 Large animals

10.1.1 CLN8 disease in dogs

The English Setter dog was the first described naturally occurring dog model [13] and has been extensively studied and reviewed [317]. There has been confirmation of the English Setters mutations as being in *CLN8* [318], and additional mutations in the

Australian Shepherd [319] and Alpenlandisiche Dachsbracke [320] have been identified (Table 1). None of these models have been used recently.

10.2 Rodents

The mouse homolog of CLN8 shares 84% amino acid identity with human CLN8 (CLUSTALW (2.1) Multiple Sequence Alignment on Ensembl (www.ensembl.org)). Mouse *Cln8* is ubiquitously expressed in various tissues, and particularly, upregulated in the developing brain [321].

A homozygous *Cln8* mutation causes a naturally occurring mouse NCL (*mnd*) [22-25]. The *mnd* mouse develops typical NCL pathology, such as autofluorescent storage material accumulation, gliosis, neurodegeneration and retinal degeneration [23, 322, 323]. Also, autofluorescent storage material accumulation and lysosomal abnormality are observed in peripheral tissues particularly in the liver [322]. The *mnd* mouse exhibits motor coordination impairment and decreased learning and memory [24, 324-326]. The *mnd* mouse is thus a valuable model for studying CLN8 functions and CLN8 disease. Several studies have suggested pathways that might be involved in CLN8 disease progression. ER stress and mitochondrial deficits are found in the *mnd* brain at pre-symptomatic stages [327, 328]. A recent study employing the *mnd* mouse model and *in vitro* culture systems found that CLN8 is required for lysosomal enzyme trafficking [329]. To date, no pre-clinical therapeutic study has been performed in the *mnd* mice.

10.3 Zebrafish

The zebrafish Cln8 protein is 289 amino acids long with 54% identity with human CLN8 (CLUSTAL W (1.81) multiple sequence alignment on Ensembl (www.ensembl.org)). Cln8 is encoded by a gene on chromosome 17 which has synteny with human CLN8, ARHGEF10, KBTBD11. One zebrafish strain containing mutation in *cln8* has been generated but not yet investigated (Table 3).

10.4 Fruit fly

Drosophila has no clear homologue of *CLN8.*

10.5 Nematode

C. elegans does not have a clear homologue of the *CLN8* gene.

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10.6 Social amoeba

The *Dictyostelium* genome does not encode a homolog of human CLN8.

11 CLN10 disease

Homozygous mutations in the lysosomal enzyme Cathepsin D (CTSD) cause CLN10 disease.

11.1 Large animals

CLN10 mutations have been identified in the American Bulldog and Swedish Landrace Sheep (Table 1). In contrast to the sheep which show congenital disease [330] and complete loss of Cathepsin D (CatD) activity, the bulldog has a protracted presentation with onset around two years of age and terminal disease by 7 years and retains some CatD activity [318]. The sheep are no longer maintained as a research flock.

11.2 Rodents

The mouse homolog of cathepsin D (CTSD) shares 80% amino acid identity with human CTSD (CLUSTALW (2.1) Multiple Sequence Alignment on Ensembl (www.ensembl.org)). *Ctsd* null mice have been used to model human CLN10 disease. The CTSD deficient mice are born normally, and then from around 2 weeks of age they develop behavioural deficits (seizures and motor abnormalities) and typical pathology of NCLs (neuronal lipofuscin deposition, lysosomal accumulation of mitochondrial ATPase subunit c, microgliosis and neurodegeneration), and die at the age of P26±1 [331-333]. Apart from brain pathology, severe visceral pathology is also observed, such as destruction of intestine and lymphoid organ, which might be the main lethal factor [333]. Early functional and structural synaptic alterations are observed in the *Ctsd* null mouse, especially in the thalamus and hippocampus [334, 335]. *Ctsd* null mice also show deficient myelination and lipid metabolism in the brain [335-338]. A study delivering AAV vectors with mouse *Ctsd* into the brain of the *Ctsd* null mice shows improvement of both the CNS and visceral pathologies and prolonged lifespan until around 2 months of age when the mice die from recurrent visceral pathology [339]. Surprisingly, the lethal recurrent visceral pathology was not accompanied by development of any NCL-like CNS pathology [339]. Cumulatively, CTSD appears to play some vital roles in the periphery in mice. It calls into question whether it is equally important for humans, and if so, preclinical work of gene therapy via systemic delivery may be necessary.

11.3 Zebrafish

Chromosome 14 harbours the zebrafish *ctsd* gene in a region without synteny with the human. The single homolog encoded by this gene is 59% identical to human Cathepsin D (CTSD) (CLUSTAL W (1.81) multiple sequence alignment on Ensembl (www.ensembl.org)). mRNA is maternally deposited and becomes stronger in the brain and yolk syncytial layer as development progresses [120, 340]. In the adult it is expressed in many tissues, including strong expression in the brain [341]. The Ctsd protein was found in the synaptosome compartment in central synapses of adult zebrafish, similar to the situation in mouse where Ctsd is found in higher levels in the synaptosome compared to post-synaptic densities [121]. Processing of zebrafish Ctsd is slightly different from CTSD in humans and this may be relevant to the interpretation of some studies [342].

Antisense morpholinos have been injected into embryos to knock down Ctsd in zebrafish embryos and larvae [340, 343]. In 2011, Follo et al. demonstrated many phenotypes in common with zebrafish lacking Tpp1, such as lack of a swim bladder, curved tail, larger yolk sac, micropthalmia and premature death from 3 - 10 dpf using the T-MPO morpholino. They went on to demonstrate a lack of microvilli on the retinal pigmented epithelial cells, which normally express high levels of Cathepsin D, but did not define the mechanism by which this results in micropthalmia [340] . It would be interesting to compare retinal phenotypes in zebrafish lacking Tpp1, Cln3 and Ctsd and define their relationships and mechanisms in common. Given that CLN6 seems to be required in the inner nuclear layer in mice [307], there may be multiple mechanisms causing retinal degeneration.

Follo *et al*. next examined the somites and musculature in straight zebrafish lacking Ctsd (T-MPO injected). They found a reduction in myofibres and thinner myosepta [343] This phenotype was seen at 4 dpf and it is not yet clear if the phenotype is due to a defect within the muscle itself or if the phenotype is an indirect consequence of loss of Ctsd in another tissue. It is possible that abnormal, or reduced numbers of, motor neurons could result in muscle phenotypes. Alternatively, the zebrafish skeletal muscle may be uniquely sensitive to levels of Ctsd as such a phenotype has not yet been reported in the mouse [344]. One mutation exists in zebrafish *ctsd*, which has not yet been investigated for a phenotype (Table 3).

11.4 Fruit fly

CLN10 disease has been modelled in *Drosophila* by generating a loss-of-function mutant in the one cathepsin D homologue, *cathD* [345]. Mutant flies exhibit a low-level accumulation of autofluorescent storage material in the brains of ageing adult flies, there is no effect on lifespan and only a small number of apoptotic cells are detectable in the brains of older flies [345]. This mild phenotype is in contrast to the congenital onset of disease in human CLN10 disease patients and is surprising given that scRNA-seq indicates strong expression of *cathD* in neurons and glia throughout the adult fly brain. The visual system of *cathD* mutant flies do display a low level of vacuolation, indicative of degeneration. This phenotype was used as the basis of a small-scale candidate screen searching for genes capable of enhancing or reducing vacuolation, which identified a potential role in endocytosis, oxidative stress responses and cholesterol trafficking [346]. The mild phenotype of the *cathD* mutant flies has also proven useful to demonstrate the importance of lysosomal function in the pathology of late-onset forms of neurodegeneration: both α-synuclein and Tau pathology is enhanced dramatically in a *cathD* mutant background [347, 348].

11.5 Nematode

Several aspartic-type endopeptidases are encoded in the *C. elegans* genome, but the closest homologue of human CTSD (cathepsin D) is *asp-4*. Based on GFP fusion protein analysis, ASP-4 protein localises to the lysosome and is highly expressed in the worm intestine, although lower level expression in various other cell types, including neurons, is evident [349]. To date, there are no reports claiming *asp-4* to be a worm model of CLN10. Nevertheless, *asp-4* has been shown to play important roles in neurodegeneration. Early work established that the non-apoptotic, necrotic cell death of mechanosensory neurons induced by various neurodegenerative triggers was

dependent on the activity of just two aspartyl proteases: ASP-3 and -4. RNAi knockdown of *asp-4* was shown to suppress degeneration of mechanosensory neurons, whereas overexpression of ASP-4 exacerbated neurodegeneration [349, 350]. In contrast, RNAi knockdown of *asp-4* has been shown to exacerbate transgenic α-synuclein accumulation while overexpression is protective against α-synuclein-induced dopaminergic neuron degeneration [351]. It seems, therefore, that *asp-4* is an important regulator of neurodegeneration that can act in a positive or negative manner depending upon the context. Further work on *asp-4* as a potential model for CLN10 disease is clearly warranted.

11.6 Social amoeba

The *Dictyostelium* homolog of human CTSD is a 383 amino acid, 41 kDa protein (CtsD) that localizes throughout the endocytic pathway, including phagosomes, macropinosomes, and lysosomes [80, 352-354]. Within a 359 amino acid region of similarity between CtsD and CTSD, 47% of the amino acids are conserved and 66% are positive matches [62]. CtsD is secreted by *Dictyostelium* cells during starvation, which is consistent with the presence of a signal peptide for secretion in both the *Dictyostelium* and human proteins [78]. The expression of *ctsD* is highest during growth [83]. *ctsD* expression decreases dramatically during the first 4 hours of starvation and then increases between 4 and 8 hours [83]. Expression then steadily decreases during the mid-to-late stages of development [83]. This expression profile suggests that CtsD may function primarily during growth and aggregation. *ctsD*-deficiency has no obvious effects on *Dictyostelium* growth or development, except for a slight delay in aggregation, which is consistent with the secretion of CtsD during this stage of the life cycle [78, 354]. Insight into the function of CtsD in *Dictyostelium* has been obtained through indirect studies. For example, *ctsD* expression is downregulated 10-fold in cells lacking the DNA-binding protein serum response factor B (SrfB) [355]. *srfB*-deficiency in *Dictyostelium* reduces proliferation, impairs cytokinesis, increases pinocytosis, and causes aberrant aggregation [355]. These phenotypes, coupled with the dramatic reduction in the expression of *ctsD* in *srfB*$^-$ cells, the delayed aggregation of *ctsD*$^-$ cells, and the secretion of CtsD during the early stages of multicellular development, suggests that CtsD is required for optimal aggregation in *Dictyostelium*. In addition, these findings are supported by work in mammalian cells that reported reduced migration of *CTSD*-deficient cells due to the abnormal organization of cytoskeletal elements [356]. In human cells, this abnormality is predicted to impact neurogenesis, neuronal migration, and the maintenance of neuronal polarity and shape [356]. Work in *Dictyostelium* has also linked CtsD function to bacterial killing and cell death. When *Dictyostelium* cells are infected with *Salmonella typhimurium*, the agent of food-borne gastroenteritis, they activate a defense response that upregulates the expression of cysteine proteases, including *ctsD* [357]. These observations are consistent with studies in mice that have linked CTSD function to the innate immune response against *Listeria monocytogenes* infection [358]. CtsD in *Dictyostelium* is also speculated to play a role in staurosporine-induced cell death, as well as oxidative stress-induced cell death [359, 360]. In line with these results, CTSD function has been linked to staurosporine-induced apoptosis in human fibroblasts and oxidative stress-induced cell death in human cancer cells [361, 362]. In total, these findings indicate that the function of human CTSD is likely conserved in *Dictyostelium* and future work in this model

organism may be able to provide new insight into the function of CTSD in human cells and how mutations in *CTSD* cause CLN10 disease.

12. CLN11 disease

CLN11 disease is caused by homozygous mutations in the soluble protein Granulin (GRN).

12.1 Large animals

There are no large animal models of CLN11 disease.

12.2 Rodents

Similar to the GRN expression in the human brain, GRN is predominantly expressed by neurons and microglia in the adult mouse brain [363-365]. A *Grn* knockout mouse line was initially generated to study the role of progranulin in mouse sexual dimorphic behaviours[366]. Further research showed that aged *Grn* knockout mice displayed extensive autofluorescent lipofuscin accumulation and gliosis by light microscopy and abundant rectilinear profiles by electron microscopy in brain neurons, which is typical of NCLs [363, 367]. Such findings were also observed with the use of other independently generated *Grn* knockout mouse lines [368, 369]. Transcriptomic analysis revealed altered expression profiles of lysosomal genes in the brain of young adult *Grn* knockout mice [370, 371], suggesting lysosomal dysregulation as an early event in the mouse *Grn* null brain. Lipidomic analysis of the brain tissue from *Grn* knockout mice and from human patients with heterozygous-*GRN*-mutation-associated frontotemporal lobar degeneration (FTLD) showed specific lipidomic profiles distinct from healthy controls and other neurodegenerative patients without *GRN* deficiency [370], which suggests one of the potential mechanisms underlying GRN-related neurodegeneration and also indicates some extent of comparability between the human *GRN* deficient brain and the *Grn* knockout mouse brain. However, distinct neurodegeneration is not evident in the *Grn* knockout mice, except for appearance of vacuolation with ageing in the hippocampal CA2-3 regions and the habenular nucleus of the Kayasuga *et al.*'s mouse model that might suggest focal neuronal loss [363, 372]. Significant neuronal loss in the hippocampus was detected in the aged *Grn* knockout mice generated by Petkau *et al.*, but it appeared to be mouse strain dependent and only present in the mice on the B6 background [373]. Behaviourally, although deficits were reported in *Grn* knockout mice, such as increased aggression, altered social behaviour and learning impairment [366, 373, 374], results are to some extent variable and still need further confirmation. It seems, therefore, that mouse models have somewhat limited benefits for modelling the progression of CLN11 disease, and thus development of large animal models that share more similarities with humans may be necessary.

Progressive vision loss due to retinal dystrophy is one of the clinical features in CLN11 patients. Retinal changes were thus studied in the *Grn* knockout mice. The adult *Grn* knockout mice generated by Kayasuga et al. exhibited significant retinal ganglion cell loss and retinal astrocyte activation, which was already observed as early as P9 [375]. Moreover, increased retinal autofluorescence, thinning of the outer nuclear layer and reduced rhodopsin (rod photoreceptor marker) were also found in

these mice [376, 377]. With the use of an independent mouse line [364], progressive retinal lipofuscinosis and retinal ganglion cell loss were also detected [378, 379]. Therefore, the *Grn* knockout mouse model might be a pre-clinical tool for developing therapies against retinal abnormalities of CLN11 disease that could somewhat improve the life quality of patients, though further retinal functional analysis and visual assessment of these mice need characterisation.

As GRN is highly expressed by both neurons and microglia, one interesting question is whether microgliosis contributes to CLN11 disease pathogenesis or whether it is just secondary to neuronal pathology. In a study investigating the effects of GRN deficiency on acute MPTP-induced neurotoxicity in the mouse brain, *Grn* knockout accelerated the neuronal loss induced by MPTP treatment, which is replicated by microglial-specific conditional *Grn* knockout [364]. The mechanism may involve a microglial phenotype induction towards a more pro-inflammatory state and subsequent enhanced neuronal death [364]. Additionally, microglial lysosomal function impairment occurs earlier than the lysosomal abnormalities in other cell types in the *Grn* knockout mouse brain, indicating a vulnerability of microglia to PGRN deficiency [380]. Thus, microglial GRN appears to play an important role in regulating microglial functions. However, the exact contribution of such role to the disease process of CLN11 still need further study. Interestingly, mice with conditional *Grn* knockout selectively targeting either neurons or microglia do not replicate the typical NCL pathology that the constitutive *Grn* null mice have shown [381, 382], indicating requirement of complete GRN depletion for CLN11 disease development and probably a non-cell autonomous manner of GRN function.

GRN gene therapy has been studied pre-clinically on the *Grn* knockout mouse model. A study employing intracranial (intraparenchymal) injection of AAV vectors with *GRN* into *Grn* knockout mice at 10-12 months of age, when they have already developed the NCL pathology, showed promising improvements of neuronal lipofuscin accumulation, microgliosis and lysosomal dysfunction in various brain regions, despite a very limited transduction area [368]. However, another recent study on *GRN* gene therapy via AAV intraventricular injection on the *Grn* knockout mice showed that *GRN* overexpression caused delayed dramatic hippocampal neurodegeneration accompanied by marked T-cell infiltration that preceded the neurodegeneration [372]. The hippocampal damage did not seem to be due to the viral vector injection or overexpressing a transgene in mice null of that gene, as there was no evident toxicity caused by AAV-*eGFP* injection into the *Grn* knockout mice or AAV-*SGSH* injection into an *Sgsh* knockout mouse model. So it might be a direct effect of excessive GRN expression and/or corresponding adaptive immune response. Such toxicity was not reported by Arrant et al.'s study we discussed earlier [368]. Notably, in that study the AAV-*GRN* vector injection induced a strong immune response at the injection site and systemic production of anti-GRN antibody in the mouse plasma, though no brain damage was observed [368]. Given that in Arrant *et al*.'s study the mice were harvested only 8-10 weeks after AAV injection [368] but in Amado *et al*.'s study the neuronal damage occurred with a delay relative to AAV injection [372], it is difficult to conclude that the *GRN* gene therapy and corresponding immune response induced in Arrant et al.'s study would have no adverse effects in the long term. In the case of CLN11 disease research, further study is definitely needed to investigate functions of GRN. *In vitro* models may be particularly useful for studying GRN functions in regulating immune cells and *GRN*-overexpression-induced neuronal toxicity. More broadly, the importance of long-term

tracking and safety assessment is highlighted again in preclinical work of gene therapy.

12.3 Zebrafish

The zebrafish genome contain two homologs of *GRN* with multiple granulin domains, as seen in the human gene, and two homologs with far less granulin domains. *grna* encodes a protein that has 12 granulin domains and *granb* encodes a protein that has 9 granulin domains, whereas *grn1* and *grn2* encode only 1.5 granulin domains each [383]. It is therefore not expected that grn1 or grn2 is the homolog of human GRN. *grna* on chromosome 3 is in a small syntenic region containing just human *GRN* and *IFI35*. It encodes a 1049 amino acid protein which has only 18% identity at the protein level (CLUSTAL W (1.81) multiple sequence alignment on Ensembl (www.ensembl.org)). The mRNA is predominantly expressed in the intermediate cell mass, which houses precursors of blood and immune cells (including microglia) in the developing embryo, in spinal cord motor neurons [384-386], and in the brain and many tissues in the adult [384]. *grnb* mRNA expression is restricted to the brain in embryos and is found in the various tissues including the brain in the adult [384]. The gene is located on chromosome 24 where there is synteny with the human chromosome synteny with the human chromosome encompassing GRN, MAP3K14, ARL17A, ARL17B, GRB7 and STCIN1. The encoded Grnb protein is 729 amino acids long and it has 25% identity at the protein level (CLUSTAL W (1.81) multiple sequence alignment on Ensembl (www.ensembl.org)). Wang *et al.* examined the structure of modules within the zebrafish Grn proteins and found that only Grna and Grnb contained modules that folded as expected. On testing the ability of a module from Grna, it was found to promote survival of neuronal cells in NSC 34 cells, but the module from Grnb was not tested [387]. It is difficult to determine, therefore, which, if any, of Grna and Grnb might be the direct homolog of human GRN.

Several papers report the effects of knockdown of Grna and Grnb. Most recently Solchenberger *et al*. reported a lack of phenotypes, after extensive investigations, in maternal zygotic *grna-/-;grnb-/-*, as well as zygotic double mutants and single mutants [383]. This somewhat contradicted previous studies where phenotypes were seen in motor axons and muscle cell precursors using antisense morpholino oligonucleotides against *grna* [385, 388, 389]. Walsh *et al.* also saw cells stuck in the cell cycle during retinal neurogenesis after injection with *grna* morpholinos [386] It is tempting to explain this by suggesting that the morpholinos cause off-target effects, but this is unlikely as the short motor axon phenotype can be rescued by injection of *grna* mRNA [385, 388]. Therefore, it may be that there is a compensatory mechanism that is only induced by the mutation. Further studies will help to distinguish the true roles of zebrafish Grna and Grnb, but this means that at the moment we are not completely satisfied that we have generated a suitable model of CLN11 disease in zebrafish. Four further mutants for *grna* and two for *grnb* currently exist and await description (Table 3).

Further experiments have tested the protective role of Grna. *grna* mRNA injected into embryos engineered to have altered levels of TDP-43, FUS and Smn1 [385, 388, 390] rescue the TDP-43 and FUS induced phenotypes.

12.4 Fruit fly

Drosophila has no clear homologue of the *GRN* gene.

12.5 Nematode

The *C. elegans* progranulin orthologue, *pgrn-1*, encodes a protein with three predicted granulin domains, compared with the 7.5 granulin domains in human progranulin [391]. Analysis of fluorescent transcriptional reporters indicates that *pgrn-1* gene expression occurs in neuronal and intestinal cells throughout larval development and into adulthood. In contrast, a translational reporter using a fluorescent protein fused to PGRN-1 was not localised to specific tissues, suggesting that the worm protein is secreted [391]. Null mutants of *pgrn-1* appear superficially wild type and have a normal lifespan, but exhibit a 20% reduction in fecundity as measured by the total number of progeny produced. More importantly, worms lacking progranulin exhibited a more rapid clearance of apoptotic cells, an effect that could be partially rescued by reintroduction of the wild type *pgrn-1* gene [391]. Such a defect in programmed cell death kinetics could potentially contribute to neuronal loss over the lifetime of human progranulin-mutation carriers. Although a neurodegenerative phenotype was not observed in *C. elegans pgrn-1* mutants, this may be because adult worms do not exhibit programmed cell death in somatic tissues, unlike vertebrates. Interestingly, *pgrn-1* mutants are resistant to some (heat, osmotic and endoplasmic reticulum stress) but not all environmental stressors, a phenotype shared by mutations in other genes affecting distinct aspects of the apoptotic process [392]. A neuroprotective role for *pgrn-1* that may relate more obviously to CLN11 disease is suggested by the observation that the PGRN-1 acts downstream of TDP-43 in a pathway that ameliorates the toxic effects of polyglutamine protein expression in a worm Huntington's disease model [393]. A further link to TDP-43 comes from the observation that heterozygous *pgrn-1* mutants exhibited worsened TDP-43 toxicity, although homozygous *pgrn-1* mutants did not [394]. Intriguingly, transgenic expression of granulins 2 and 3 (but not granulin 1) exacerbated the toxicity of co-expressed TDP-43, as indicated by defects in behaviours including locomotion and fecundity [394]. A recent pre-print has claimed that granulins selectively interact with the aspartyl protease ASP-3/cathepsin D in *C. elegans*, impairing its enzymatic activity and hence lysosome function [395]. This direct connection between two proteins implicated in distinct CLN diseases revealed in *C. elegans* has potentially important mechanistic and therapeutic implications.

12.6 Social amoeba

The *Dictyostelium* homolog of human PGRN, Grn, shares sequence similarity with human GRNs 1–7 [66]. The *grn* gene encodes a 130 amino acid, 14 kDa protein. Within a 47 amino acid region of similarity between Grn and PGRN, 55% of the amino acids are conserved and 68% are positive matches [62]. Expression of *grn* is highest during growth [83]. Upon starvation, expression decreases dramatically reaching its lowest level during the mid-to-late stages of multicellular development [83]. This expression profile suggests that Grn likely functions during growth and may not be essential for aggregation or differentiation. Future work that generates and characterizes a *grn*-deficient *Dictyostelium* cell line may be able to provide insight into the primary function of PGRN in human cells and how mutations in this gene cause CLN11 disease.

13. CLN12 disease

CLN12 disease is caused by homozygous mutations in the transmembrane protein ATPase Cation Transporting 13A2 (ATP31A2).

13.1 Large animals

The only confirmed cases of CLN12 disease in large animals was found in Tibetan terriers [396, 397]. While mutations in CLN12, encoding ATP13A2 can cause Kufor-Rakeb syndrome, an early onset Parkinson's disease, the dogs display a late-onset NCL, also now confirmed in human cases [398].

13.2 Rodents

Mouse *Atp13a2* is mapped to chromosome 4 [399], and the encoded protein shows >80% identity with human ATP13A2 (CLUSTALW (2.1) Multiple Sequence Alignment on Ensembl (www.ensembl.org)). It is expressed in all mouse tissues and shows particularly high expression in the brain at the transcriptional level [399]. Two research groups have generated and characterized *Atp13a2* knockout mouse lines independently [400-402]:both lines develop and reproduce normally and display typical NCL features at old age, such as motor deficits and neuronal lipofuscin accumulation and gliosis throughout the CNS. Another mouse line with brain-specific conditional *Atp13a2* knockout also confirms that ATP13A2 deficiency in the mouse brain resembles late-onset behavioural phenotypes (motor impairments) and typical NCL pathological characteristics (neuronal lipofuscin accumulation positive for mitochondrial ATPase subunit c) [403]. Interestingly, astrocytosis is found to occur much earlier (1 month of age) compared to other pathology such as lipofuscin accumulation (12 months of age) and the behavioural deficits (18 months of age) in the *Atp13a2* null mouse [400]. Particularly, heterozygous *Atp13a2* knockout mice show high-level astrogliosis throughout the CNS without robust lipofuscinosis [401], suggesting a potential independent role of ATP13A2 in astrocyte function. However, although it has been suggested that astrocyte ATP13A2 deficiency induces accelerated astrocyte inflammatory cytokine release *in vitro* [404], contribution of astrocyte ATP13A2 in CLN12 disease is still unclear and further study is needed to investigate the ATP13A2-deficiency-induced *in vivo* astrocyte changes at subcellular/molecular levels.

As ATP13A2 mutations are also involved in Kufor-Rakeb syndrome (KRS, a genetic form of Parkinson's disease), researchers investigated alpha synuclein aggregation and dopaminergic pathology in the *Atp13a2* null mice. In contrast to the NCL pathology, the ATP13A2 deficient mice do not significantly show CNS alpha synuclein pathology or dopaminergic neuronal changes in the substantia nigra [400-402], suggesting the *Atp13a2* knockout mouse alone may not be an ideal model to recapitulate Parkinson's disease. However, with respect to CLN12 disease, *Atp13a2* null mice clearly provide a valuable mammalian model for investigating the disease pathogenesis and future pre-clinical therapeutic studies.

13.3 Zebrafish

The zebrafish encodes a homolog of ATP13A2 on the *atp13a2* gene located on chromosome 23. Atp13A2 is 1170 amino acids, with 51% identity with human ATP13A2 (CLUSTAL W (1.81) multiple sequence alignment on Ensembl (www.ensembl.org)). The small region of synteny encompasses ATP13A2 and SDHB only. Expression of *atp13a2* mRNA is initially everywhere at 12 hpf, but soon becomes restricted to the brain [120, 405]. Knockdown using antisense morpholino oligonucleotides results in reduced embryonic survival, small eyes, a curved body, a large yolk sac and impaired locomotion, which are all phenotypes in common with Tpp1 [28] and Ctsd [340] knockdown zebrafish. However, others failed to generate a phenotype with morpholinos against ATP13A2 [213]. Five mutant alleles exist, which have not yet been investigated (Table 3).

13.4 Fruit fly

Drosophila has a clear homologue of ATP13A2 encoded by the *anne boleyn (anne)* gene (CG32000). scRNA-seq data indicate *anne* is highly expressed in most neurons, potentially all. Studies of *anne* are limited. However, knockdown of *anne* in neurons in the *Drosophila* CNS leads to accumulation of storage material in lysosomes that worsens with age, accompanied by increased insoluble ubiquitinated proteins. These phenotypes can be suppressed by overexpression of HDAC6 [406].

13.5 Nematode

catp-6 is the closest *C. elegans* homologue of human ATP13A2, although *catp-5* and *catp-7* also show significant homology. It seems there is some redundancy in function between these three paralogues, as although single null mutants are viable, *catp-6*; *catp-5* and *catp-6*; *catp-7* double mutants are sterile [407]. Studies using fluorescent proteins fused to CATP-6 suggest that the protein is expressed in various cell types, including neurons, where it localises to cortical vesicular structures [407]. Null mutant *catp-6* worms are slow growing, exhibit uncoordinated locomotion, and have significantly reduced fecundity as determined by the number of progeny reaching the L4 larval stage within 2 days [407]. Transgenic expression of *catp-6* in dopaminergic neurons ameliorated the degeneration of these cells induced by co-expressed α-synuclein, whereas RNAi knockdown of *catp-6* exacerbated dopaminergic neurodegeneration [408]. These findings are clearly relevant for Parkinson's disease, given its established link to mutations in ATP13A2/PARK9, but may also have implications for CLN12 disease.

13.6 Social amoeba

The *Dictyostelium* homolog of human ATP13A2 is Kil2, a P-type ATPase that contains 10 putative transmembrane domains [62, 409]. The 1158 amino acid, 131 kDa Kil2 protein is encoded by the *kil2* gene. Within a 1089 amino acid region of similarity between Kil2 and ATP13A2, 35% of the amino acids are conserved and 53% are positive matches [62, 409]. Like other NCL protein homologs in *Dictyostelium*, Kil2 is present in the macropinocytic pathway [80]. Expression of *kil2* increases steadily during development reaching peak levels during fruiting body formation, suggesting that the protein plays a role in differentiation [83]. Kil2 localizes to the phagosomal membrane and *kil2*-deficiency impairs the ability of cells

to kill ingested *Klebsiella pneumoniae* bacteria [409, 410]. However, this defect appears to be species-specific, since *kil2*$^{-}$ cells can kill several other species of bacteria without impairment (e.g., *B. subtilis* and *P. aeruginosa*) [409]. The impaired ability of *kil2*$^{-}$ cells to destroy ingested *Klebsiella pneumoniae* can be restored by supplementing the medium with magnesium [409]. These results suggest that Kil2 functions as a magnesium pump to maintain the optimal concentration of magnesium in phagosomes, thereby enabling the activity of phagosomal proteases [409]. Recent work also indicates that the role of Kil2 in killing *Klebsiella pneumoniae* may involve vacuolar protein sorting protein 13F (Vps13F) [411]. Interestingly, VPS proteins have been linked to the function of NCL protein homologs in yeast [412, 413]. Finally, the function of Kil2 has also been linked to the predation of *Dictyostelium* amoeba on yeast [414]. In mammalian cells, ATP13A2 localizes to the lysosome and is speculated to function as a biometal transporter [415, 416]. As a result, the protein has been linked to processes that rely on biometal transport, such as autophagy [406, 417]. In total, work on Kil2 function supports the putative role of human ATP13A2 in maintaining biometal homeostasis in lysosomes and indicates that the future study of Kil2 in *Dictyostelium* may reveal novel insight into the function of ATP13A2 that is lost in patients with CLN12 disease.

14. CLN13 disease

Homozygous mutations in the lysosomal enzyme Cathepsin F (CTSF) cause CLN13 disease.

14.1 Large animals

There are no large animal models of CLN13 disease.

14.2 Rodents

Mouse cathepsin F shares >80% identity with human human Cathepsin F [418]. The encoding gene was identified to be on mouse chromosome 19 and expressed ubiquitously in various mouse tissues [418]. Widespread expression of mouse cathepsin F in the CNS and its lysosomal localization was also confirmed [419]. Before mutation in human *CTSF* was identified as the pathogenic gene in CLN13 disease, a *Ctsf* null mouse model had been generated by homologous recombination, exhibiting a late-onset NCL-like phenotype [419]. The *Ctsf* null mouse does not show obvious phenotypes (progressive hindlimb weakness and motor coordination impairment) until 12-16 months of age, and dies within the following 4-6 months [419]. Neuronal autofluorescent storage material accumulation accompanied by astrogliosis and neurodegeneration was observed in the CNS of the *Ctsf* null mouse model [419, 420]. Electron microscopic analysis revealed lamellated inclusions in the spinal cord neurons of *Ctsf* null mice [419, 420], though no increase was found in the levels of mitochondrial ATPase subunit c [419, 420]. Future work employing this mouse model may provide important information on *in vivo* functions of cathepsin F and may reveal biological mechanisms underlining how CTSF deficiency results in CLN13 disease progression.

14.3 Zebrafish

Zebrafish Ctsf is 473 amino acids, 47% identical to the human homolog (CLUSTAL W (1.81) multiple sequence alignment on Ensembl (www.ensembl.org)) and encoded by the *ctsf* gene on chromosome 14. There is only one *ctsf* gene in zebrafish and it sits in a very small region of synteny that is made of TRPT1 and CTSF. The mRNA is known to be expressed in the adult retina [421] and in the brain and spinal cord during development [120]. Five mutations exist but a phenotype has not yet been reported (Table 3).

14.4 Fruit fly

The *Drosophila* Ctsf homologue is encoded by CG12163. It is expressed widely throughout the CNS but has not yet been studied in the context of the NCLs. Multiple lines are available to study CG12163, including UAS-RNAi lines and potential insertional mutants (see flybase.org).

14.5 Nematode

The predicted product of the *C. elegans* temporarily assigned gene *tag-196* displays 49% amino acid identity to Cathepsin F over a 300-amino-acid stretch among its 487-amino-acid full-length sequence. According to WormBase, *tag-196* is expressed in various tissues, including muscle, intestine, the reproductive system and neurons. Although four different predicted null mutant alleles are available, there are no published data on the phenotypes of these mutants, so the physiological function of *tag-196* in *C. elegans* is unknown.

14.6 Social amoeba

A previous study identified cysteine proteinase A (CprA) as the likely homolog of human CTSF in *Dictyostelium* [62]. The *cprA* gene encodes the 343 amino acid, 39 kDa CprA protein. Within a 340 amino acid region of similarity between CprA and CTSF, 37% of the amino acids are conserved and 55% are positive matches [62]. CprA is speculated to play a role in the digestion of proteins to provide amino acids for developing cells [422]. *cprA* is not expressed during growth, but expression, which is induced by extracellular cAMP, increases dramatically during the early stages of development [83, 423]. *cprA* has also been identified as a pre-stalk specific gene [424]. CprA contains a signal peptide for secretion and is secreted during *Dictyostelium* development, which is consistent with the secretion of CTSF in humans [78, 425]. Indirect studies have provided insight into the function of CprA in *Dictyostelium*. For example, the expression of *cprA* is induced during hypertonic stress suggesting that the protein plays a role in osmoregulation [426]. Expression is also upregulated during starvation in response to *rpkA*-deficiency [427]. Receptor phosphatidylinositol kinase A (RpkA) is a seven transmembrane G-protein coupled receptor (GPCR) that localizes to phagosomes and is involved in the phagocytosis of infectious bacteria [428]. Importantly, this work is consistent with the proposed role of CTSF in cell immunity in mammalian cells [429]. Also, *rpkA*-deficiency impairs aggregation due an inability to respond to extracellular cAMP [427]. Given the increased expression of *cprA* in *rpkA*$^{-}$ cells, and the dramatic increase in *cprA*

expression upon starvation, these findings indicate that CprA may play a role in cAMP-mediated chemotaxis and aggregation.

While CprA has been identified as the likely homolog of human CTSF in *Dictyostelium*, there are several additional cysteine proteases that share sequence similarity with the human protein. Among these proteins are cysteine proteinase B (CprB, 376 amino acids, 33% conserved amino acids and 47% positive matches within a 342 amino acid region of similarity) and the uncharacterized protein DDB0252831 (352 amino acids, 37% conserved amino acids and 52% positive matches within a 339 amino acid region of similarity, encoded by gene *DDB_G0291191*). Based on their size and similarity, either of these proteins could also be the *Dictyostelium* homolog of human CTSF. Like CprA, both proteins are secreted by *Dictyostelium* cells during starvation [77, 78]. Protein DDB0252831 also localizes to endocytic vesicles and the macropinocytic pathway [80, 430]. The expression profile of *cprB* is almost identical to that of *cprA* [83]. Like *cprA*, the expression of *cprB* is induced by extracellular cAMP and *cprB* is characterized as a pre-stalk specific gene [423, 424]. However, unlike *cprA* and *cprB*, gene *DDB_G0291191* (protein DDB0252831) is expressed at high levels during growth [83]. Expression then decreases dramatically upon starvation, reaching its lowest level after 8 hours of development, and remains low during the mid-to-late stages of multicellular development [83]. This expression profile suggests that protein DDB0252831 functions primarily during growth, which is supported by previous work that linked the protein to the cellular response to bacterial pathogens [431]. Taken together, these findings indicate that protein DDB0252831 may function as the dominant CTSF-like protein during growth, while CprA and CprB may both function as the primary CTSF-like proteins during development. Gene-deficiency models have not yet been generated for *cprA*, *cprB*, or gene *DDB_G0291191* in *Dictyostelium*. Therefore, future work to generate and characterize cells lacking these genes should provide insight into their functions, and hopefully shed light on which of these genes is the true homolog of human CTSF in *Dictyostelium*.

15. CLN14 disease

Homozygous mutations in Potassium Channel Tetramerization Domain Containing 7 (KCTD7) cause CLN14 disease. Sequence analysis suggests that the protein may function as a voltage-gated potassium channel.

15.1 Large animals

There are no large animal models of CLN14 disease.

15.2 Rodents

Mouse *Kctd7* at chromosome 5 encodes the mouse homolog of human KCTD7. The protein sequence of mouse KCTD7 is ~97% identical to human KCTD7 (CLUSTALW (2.1) Multiple Sequence Alignment on Ensembl (www.ensembl.org)). It has been shown that KCTD7 is widely expressed in both developing and adult mouse brains, especially in the hippocampus, cerebral cortex and cerebellum [432, 433]. However, the *in vivo* function of KCTD7 in the mouse brain is still not clear, and no murine models have yet been generated for CLN14 disease.

15.3 Zebrafish

Chromosome 10 of the zebrafish harbours the sole zebrafish homolog of KCTD7. Synteny extends from CUX1, through to KCTD7 and RABGEF. The encoded protein is 339 amino acids, with 62% identity with the human protein (CLUSTAL W (1.81) multiple sequence alignment on Ensembl (www.ensembl.org)). No phenotype has yet been reported for the four mutations that exist (Table 3) and there are no studies so far showing mRNA or protein localisation.

15.4 Fruit fly

Drosophila has no clear homologue of KCTD7.

15.5 Nematode

The *C. elegans* genome encodes numerous predicted proteins with significant similarity to human KCTD7. It is unclear which, if any, of these represents the KCTD7 orthologue and therefore might represent a potential model for CLN14 disease.

15.6 Social amoeba

A previous study reported that the *Dictyostelium* homolog of human KCTD7 is Kctd9, which is a 488 amino acid, 53 kDa protein that is encoded by the *kctd9* gene [62]. Kctd9 contains four penta-peptide repeats, a double-courtin domain, and is highly similar to potassium channel tetramerization domain-containing proteins found in vertebrates [66]. Within a 96 amino acid region of similarity between Kctd9 and KCTD7, 38% of the amino acids are conserved and 58% are positive matches [62]. The expression of *kctd9* decreases during the early stages of development and reaches its lowest level after 4 hours of starvation [83]. Expression then increases significantly, reaching its peak level by 12 hours, after which time expression decreases steadily during the mid-to-late stages of development [83]. In addition to Kctd9, there are three additional proteins encoded by the genome that are similar to human KCTD7 (DDB0238663, DDB0346929, and DDB0347398 encoded by genes *DDB_G0269760*, *DDB_G0285861*, and *DDB_G0269022*, respectively). These three proteins are similar in sequence to human KCTD7 (33-38% conserved amino acids and 49-60% positive matches within a 95-99 amino acid region of similarity), but unlike Kctd9, are more similar in size to human KCTD7 (346-366 amino acids vs. 488 amino acids for Kctd9 vs. 289 amino acids for KCTD7). Future work on Kctd9 and the three uncharacterized proteins may shed light on which protein is the true *Dictyostelium* homolog of human KCTD7. Once this is established, *Dictyostelium* can be used a model system to study how mutations in human *KCTD7* cause CLN14 disease.

16. Concluding remarks

This review presents an update on the range of multicellular model organisms and disease models being used for NCL research, in the hope that this will stimulate

researchers to collaborate and employ the most suitable model(s) for their study. This is particularly important before a therapeutic enters clinical trial, where positive outcomes from studies using more than one organism will reduce the risk of failure.

It is evident that the choice of model organism to be used for a study is first determined by the existence of a clear homologue, particularly when considering using an invertebrate. However, this implies that other parts of a pathway that is conserved in humans and vertebrates may be missing in invertebrates. Nonetheless, the power of these organisms for mechanistic studies is undeniable.

The zebrafish is being increasingly used and embryonic/larval disease models such as those for CLN2 and CLN3 disease are the only vertebrate models that can be cheaply, quickly and easily used for drug discovery. Certainly, more NCL disease models could be generated and validated in the zebrafish, and many mutants exist that have not yet been examined for an NCL-like phenotype. Issues with zebrafish include identifying the correct homologue to target when more than one exists, or the possible need to knock down both homologues. In this organism, knock-down induced using different means seems to result in different phenotypes (if any) – further research is needed to uncover why this is, but one tantalizing possibility is the existence of one or more novel compensation mechanisms that suppresses phenotypes induced by certain types of mutation. This is also true in *Dictyostelium* where multiple homologs exist for some of the NCL proteins (e.g., homologs of TPP1 and CTSF). One caveat to using *Dictyostelium* as a biomedical model is the limited number of cell types in the organism, which may ultimately limit the translation of findings to specific tissues or organs in mammalian systems. Nonetheless, the presence of 11 homologs of NCL proteins in *Dictyostelium*, coupled with genetic tractability of the organism, suggests that future work in *Dictyostelium* has the potential to shed new insight on the functions of NCL proteins in human cells. With this in mind, the *Dictyostelium* research community is very active in generating knockout models to study the functions of the various NCL protein homologs in this organism. The nematode worm, being the smallest and simplest of all animal models, provides an intermediate between zebrafish and *Dictyostelium*. Although its short lifespan and facile molecular genetics make it attractive for modeling NCLs, relatively few CLN disease genes have been investigated in detail in *C. elegans*. This is due at least in part to the lack of obvious homologues for several genes, but for those genes with clear worm orthologues, it is anticipated that new and improved *C. elegans* models will be developed in the near future.

Mouse models have been, and continue to be, the mainstay of NCL research. However, phenotypes often take a long time to develop and do not always faithfully recapitulate the cognate human disease. In such cases, initial studies using *in vivo* models with a faster disease onset or displaying phenotypes in additional relevant tissues, may be useful. For example, despite a greater need for gene therapy for CLN3 disease, this has been pioneered first in the mouse model of CLN6 disease because it has a faster onset and progression.

Large animals displaying NCL are undeniably useful for therapeutic testing, usually after initial testing on smaller organisms. Until recently, we have relied upon naturally-occurring large animal models but the recent implementation of gene editing in veterinary species is providing an increasing range of relevant large animal models.

Together, multicellular models of NCL have been, and will continue to be, crucial for understanding pathology and testing new therapies. Understanding the relative

strengths and weaknesses and comparing findings in multiple model systems will aid in clinical translation.

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Table 1. The use of existing large animal models in NCL research.

Large animal model species	Gene mutation	Examples of use for pathology/basic research	Use in pre-clinical studies
Dogs			
Dachshund	*CLN1:c.736_737 insC [86]*	Original description only	-
Cane Corso	*CLN1:c.124+1G > A [87]*	Original description only	-
Dachshund	*CLN2:c.325delC [27]*	Retinal pathology[434]	Gene therapy, enzyme replacement, Stem cells
Border Collie	*CLN5:c.619C > T [264]*	Biomarkers[264]	-
Golden Retriever	*CLN5:c934_935delAG [266]*	Original description only	-
Australian Shepherd	*CLN6:c.829T >C [435]*	Original description only	-
Chinese crested dog, Chihuahua	*CLN7:c.843delT [311]*	Original description only	-
English Setter	*CLN8:c491T >C [436]*	[437]	Carnitine supplementation [438]
Australian Shepherd	*CLN8:c.585G >A [319]*	Original description only	-
Alpenlandische Dachsbracke	*CLN8:g.30852988_30902901del [320]*	Original description only	-
Saluki	*CLN8:c.349dupT [439]*	Original description only	-
American Bulldog	*CLN10:c.597G >A [318]*	Original description only	-
Tibetan Terrier	*CLN12:c.1623delG [440, 441]*		
Sheep			
Borderdale (NZ)	*CLN5:c.571+1G>A [268]*	Primary neural cultures[291, 293], biomarkers[275, 276, 295], synaptic changes [442]	Gene therapy [270, 272]
South Hampshire (NZ)	*CLN6*	Extensive characterization since 1970s, primary neural cultures[293], biomarkers[295], glial pathology[296]	Gene therapy, minocycline drug trial [297]
Merino (Australia)	*CLN6:c.184C>T [443]*	Biomarkers[299]	-
Swedish Landrace (Finland)	*CLN10*	[330]	
Monkey	*CLN7:c.769del [18]*	Original description only	-
Cattle			
Devon (Australia)	*CLN5:c.662dupG [444]*	Original description only	-

Table 2. The available murine models in NCL research.

Disease (causative gene)	Mouse model	Original reference	Pre-clinical studies
CLN1 (*PPT1*)			
	Ppt1 knockout mouse	[95]	AAV gene therapy [115-119] Recombinant PPT1 [112, 113] Thioesterase mimicking drug [108, 109, 114]
	Ppt1 knockout mouse	[96]	-
	Ppt1 (R151X) knock-in mouse	[105]	-
	Ppt1 (R151X) knock-in mouse	[106]	Nonsense suppression therapy [106]
CLN2 (*TPP1*)			
	Tpp1 knockout mouse	[29]	AAV gene therapy [137, 151-154] Enzyme replacement [138, 156-158] Gemfibrozil [159]
	Tpp1 (R207X) knock-in mouse	[150]	-
CLN3 (*CLN3*)			
	Cln3 knockout mouse	[176, 178]	Glutamate receptor antagonist [183, 184, 445]
	Cln3 knockout mouse	[177]	-
	Cln3 $^{\Delta ex7/8}$ knock-in mouse	[175]	AAV gene therapy [204, 205] Phosphodiesterase-4 inhibitor [208] Trehalose [209]
	Cln3 reporter knock-in mouse	[173]	Carbenoxolone [169]
CLN4 (*DNAJC5*)			
	Dnajc5 knockout mouse	[245]	Overexpression of alpha-synuclein [244]
CLN5 (*CLN5*)			
	Cln5 knockout mouse	[279]	-
CLN6 (*CLN6*)			
	Naturally occurring *nclf* mouse	[19]	AAV gene therapy [307]
CLN7 (*MFSD8*)			
	Mfsd8 knockout mouse	[312, 313]	-
CLN8 (*CLN8*)			
	Naturally occurring *mnd* mouse	[22, 24]	-
CLN10 (*CTSD*)			
	Ctsd knockout mouse	[333]	Anti-inflammatory therapy [332]
CLN11 (*GRN*)			

	Grn knockout mouse	[366]	AAV gene therapy [372]
	Grn knockout mouse	[369]	-
	Grn knockout mouse	[364]	[368]
	Grn knockout mouse	[374, 446]	-
CLN12 (*ATP13A2*)			
	Atp13a2 knockout mouse	[400]	-
	Atp13a2 knockout mouse	[402]	-
	Atp13a2 conditional knockout (brain-specific) mouse	[403]	-
CLN13 (*CTSF*)			
	Ctsf knockout mouse	[419]	-
CLN14 (*KCTD7*)			
	No murine models	-	-

Table 3. Zebrafish homologues of human NCL genes, available models and known associated phenotypes.

Human Protein	Zebrafish Homologue	Zebrafish model	Molecular defect	Phenotype	Journal articles
CLN1/PPT1	Ppt1	*ppt1*^{sa9}	nonsense mutation/predicted protein truncation after exon2	not described	
		ppt1^{sa7888}	nonsense mutation/predicted protein truncation after exon 3	not described	
CLN2/TPP1	Tpp1	*tpp1*^{sa11}	nonsense mutation/predicted protein truncation after exon 3 results in loss of protein by immunofluorescnce	small eyes and head at 2 dpf, lethality by 7 dpf, SCMAS increases, apoptosis increases, reduced cell proliferation, lysotracker increases, activity increased at 3 dpf and decreased at 4 dpf	[28]
		tpp1^{sa2895}	essential splice site for exon 9	not described	
		tpp1^{sa6483}	nonsense mutation/predicted protein truncation after exon 10	not described	
		tpp1^{hu3587}	nonsense mutation/predicted protein truncation after exon 11	normal	[28]
		tpp1^{sa8389}	nonsense mutation	not described	
		tpp1 ATG/splice site MOs	splice MO affects splicing	small eyes and head at 2 dpf	[28]
CLN3	Cln3	*cln3* ^{sa20092}	nonsense mutation in exon 10	not described	
		cln3 ATG/splice site MOs	splice site MO affects splicing	small eyes and head at 2 dpf, lethality at 5 dpf, activity increased at 36 hpf and decreased at 4 dpf, astrocytosis, apoptosis increases, reduced cell proliferation, lysotracker increases, SCMAS increases, mitochondrial membrane potential altered	[210]
CLN4/DNAJC5	Dnajc5aa Dnajc5ab				
CLN5	Cln5				
CLN6	Cln6a	*cln6a*^{sa904}	essential splice site for exon 6	not described	
		cln6a^{sa10876}	nonsense mutation	not described	
		cln6a^{la016089Tg}	retroviral insertion	not described	[447, 448]

	Cln6b	*cln6b*^{sa37974}	splice site	not described	
CLN7/MFSD8	Mfsd8	*mfsd8*^{sa2646}	essential splice site for exon 5	not described	
		mfsd8^{la018118Tg}	transgenic insertion	not described	[447, 448]
		mfsd8^{sa27950}	nonsense mutation	not described	
		mfsd8^{sa42028}	nonsense mutation	not described	
		mfsd8^{sa45459}	splice site for exon 12	not described	
CLN8	Cln8	*cln8*^{sa25040}	not described	not described	
CLN10/CTSD	Ctsd	*ctsd*^{sa36610}	nonsense mutation/predicted protein varies according to transcript	not described	
		CD T-MPO	complete protein knockdown	micropthalmia (RPE microvilli affected), shorter body, swim bladder not inflated, hyper-pigmentation, impaired yolk absorption (all at 4dpf), premature death (10dpf)	[340, 343]
CLN11/GRN	Grna	*grna* ^{sa6034}	nonsense mutation/predicted protein varies according to transcript	not described	
		grna ^{sa20026}	nonsense mutation/predicted protein varies according to transcript	not described	
		grna^{sa38396}	splice site/exon affected varies according to transcript	not described	
		grna^{sa33188}	splice site/exon affected varies according to transcript	not described	
		grna^{mde33}	1bp deletion in exon 3	normal	[383]
		grna^{mde54a}	11bp deletion in exon 3	normal	[383]
		grna^{mde54b}	11bp deletion in exon 3	normal	[383]
		grna^{mde54c}	1bp insertion/11 bp deletion in exon 3	normal	[383]
		grna MO (various)	severe protein knockdown, reduction assessed by RT PCR	reduced motility, widespread disruption of CNS development, motoneuron axon truncation and premature branching, smaller myofibrils	[385, 389, 390, 449, 450]
	Grnb	*grnb*^{sa30129}	nonsense mutation in exon 15	not described	
		grnb^{sa37944}	nonsense mutation in exon 13	not described	
		grnb^{mde357a}	7bp deletion in exon 5	normal	[383]
		grnb^{mde360}	9bp insertion.13bp deletion in exon 5	normal	[383]
		grnb^{mde369}	5bp insertion in exon 5	norma	[383]
		grnb MO (various)	reduction assessed by RT PCR	reduced motor neuron axon length	[390, 451]

CLN12	Atp13a2	*atp13a2*^{sa1158}	essential splice site for exon 15	not described	
		atp13a2^{sa3236}	nonsense mutation in exon 24	not described	
		atip13a2^{sa6735}	essential splice site for exon 29	not described	
		atp13a2^{sa14250}	nonsense mutation in exon 20	not described	
		atp13a2^{sa18624}	nonsense mutation in exon 22	not described	
		atp13a2 MO	affects splicing	small eyes and head at 2 dpf, reduced activity at 7dpf	[405]
CLN13	Ctsf	*ctsf*^{sa17667}	essential splice site for exon 1	not described	
		ctsf^{sa22423}	essential splice site for exon 10	not described	
		ctsf^{sa28260}	nonsense mutation in exon 1	not described	
		ctsf^{sa35632}	nonsense mutation in exon 8	not described	
		ctsf^{sa42345}	essential splice site mutation for exon 12	not described	
CLN14	Kctd7	*kctd7*^{hg34}	5bp deletion in exon 1	not described	[452]
		kctd7^{hg35}	4bp insertion in exon 1	not described	[452]
		kctd7^{sa6178}	nonsense mutation in exon 2	not described	
		kctd7^{sa16727}	essential splice site for exon 2	not described	

Table 4. *Drosophila* homologues of human NCL genes, available models and known associated phenotypes.

Human Protein	*Drosophila* Homologue	*Drosophila* model	Molecular defect	Phenotype	Journal articles
CLN1/PPT1	*Ppt1*	*Df(1)446-20*	Small deficiency deleting Ppt1 and neighbouring loci	Accumulation of autofluorescent deposits in the CNS, reduced lifespan.	[122]
		Ppt1^{A179T}	A179T substitution	Altered vesicle dynamics at synapses.	[125]
		Ppt1^{S77F}	S77F substitution		
		UAS:DmPpt1$^{8.1}$		Disruption of the eye modified by regulators of endocytosis and synaptic development.	[123, 453]
		RNAi lines available	Overexpression under Gal4 control		
CLN2/TPP1	No homologue				
CLN3	*Cln3*	*cln3$^{\Delta MB1}$*	Deletion of 5' half of locus	Hypersensitivity to oxidative stress; under development of neurons at the neuromuscular junction.	[128, 215] [214, 215]
		UAS-Cln3	Overexpression under Gal4 control		[217]
		UAS-YFP-Cln3	Overexpression under Gal4 control	Genetic interactions with Notch and JNK signalling pathways; Oxidative stress in the eye suppressed by increasing ROS scavenging	
		YFP-Cln3	Knock-in gene fusion		
		RNAi lines available		Glia expression in the CNS; strong expression in Malpighian tubules, apical localisation in polarised tubules.	
CLN4/DNAJC5	*Cysteine string protein (Csp)*	37 alleles available	Various deletions, insertions, point mutations	Temperature sensitive locomotor defects, spasms, paralysis, progressive neurodegeneration. Alterations to the Ca^{2+} mediated endocytosis at synapses.	[249]; see flybase.org for others
		Multiple overexpression, point mutations, domain deletions and RNAi lines available			
CLN5	No homologue				

CLN6	No homologue				
CLN7/MFSD8	*CG8596*	*YFP-Cln7* RNAi lines available	Knock-in gene fusion	Expression in glia in the CNS; in the neurons in the visual system; localisation to the post-synaptic site at the larval neuromuscular junction.	[217]
CLN8	No homologue				
CLN10/CTSD	*CathD*	*cathD*^{1} RNAi lines available	Deletion of 70% of the coding sequence	Autofluorescent accumulation in the CNS, vacuolation in the retina; no effect on lifespan. Enhances pathology of Tau and a-synuclein	[345, 346] [347, 348]
CLN11/GRN	No homologue				
CLN12	*anne boleyn (anne)/CG32000*	RNAi lines Potential insertional mutants		Knockdown causes age-dependent storage material accumulates in neurons.	[406]
CLN13	*CG12163*	RNAi lines Potential insertional mutants		Not studied	
CLN14	No homologue				

Table 5. *C. elegans* homologues of human NCL genes, available models and known associated phenotypes.

Human protein	*C. elegans* gene	Gene manipulation	Molecular defect	Phenotypes reported
CLN1/PPT1	*ppt-1*	*gk131*	Δ20-100; V insertion at 100	Increased locomotion and maximum lifespan [130]
		gk134	Δ33-169; frame shift from 160	Decreased body length and locomotion on agar [130] [130]
		gk139	Δ84-200	Mitochondrial abnormalities and developmental delay [129]
		gk140	Deletion of exons 2-3	Developmental delay [129]
		RNAi	Reduced expression	Decreased median lifespan [130]
CLN2/TPP1	No homologue			
CLN3	*cln-3.1*	*pk479*	Complete ORF deletion	Decreased lifespan [221]
	cln-3.2	*gk41*	Deletion in promoter + exons 1-2	Decreased fecundity [221]
	cln-3.3	*gk118*	Deletion of exons 5-7	
		pk479; gk41;gk118	Triple deletion mutant	Decreased fecundity + lifespan [221, 222]; increased mitochondria [222]
CLN4/DNAJC5	*dnj-14*	*ok237*	Deletion of promoter + ORF	Short lifespan [253, 259], progressive sensory neuron degeneration [253]
		tm3223	Δ106-214	Short lifespan [253, 259], progressive sensorimotor dysfunction [253, 259]
		RNAi	Reduced expression	Short lifespan, progressive motor dysfunction [253]
CLN5	No homologue			
CLN6	No homologue			
CLN7/MFSD8	Y53G8AR.7 ZK550.2 F14D7.6 T28C12.1 T28C12.2 W05E10.1	RNAi	Reduced expression	Increased lifespan and paraquat resistance [316]
CLN8	No homologue			
CLN10/CTSD	*asp-4*	*ok2693*	Deletion of exons 5-6	

		RNAi	Reduced expression	Increased [351] or decreased [349, 350] neuronal cell death
		Plasmid	Overexpression	Decreased [351] or increased [349, 350] neuronal cell death
CLN11/GRN	*pgrn-1*	*tm985*	Deletion of promoter + exon 1	Altered apoptosis [391]; stress resistance [392]; TDP-43 [393, 394]
		Plasmid	Overexpression	Granulins increase toxicity of TDP-43 [394]
CLN12/ATP13A2	*catc-6*	*ok3473*	Δ280 AAs + frame shift	Slow growth, locomotion defect, reduced fecundity [407]
		RNAi	Reduced expression	Reduced dopaminergic neurodegeneration [408]
		Plasmid	Overexpression	Exacerbated dopaminergic neurodegeneration [408]
CLN13/CTSF	*tag-196*	*ok822*	Deletion of promoter + exons 1-3	
		ok823	Deletion of exons 1-3	
		ok824	Deletion of exons 2-4	
		tm612	Deletion of exons 4-5	
CLN14/KCTD7	Unclear			

Table 6. Localization and cellular processes modulated by NCL protein homologs in *Dictyostelium*

Human NCL protein	*Dictyostelium* homolog	dictyBase ID	Localization in *Dictyostelium*	Processes modulated in *Dictyostelium*
PPT1/CLN1	**Ppt1**	DDB0233890	Extracellular space [77] Macropinocytic pathway [80]	Phagocytosis [131]
TPP1/CLN2	**Tpp1A**	DDB0234303	Lysosome [164]	Autophagy [164] Pre-spore cell differentiation [164] Timing of mid-stage development [164]
	Tpp1B	DDB0306176	Golgi complex [165] Extracellular space [77, 78] Macropinocytic pathway [80]	Not known
	Tpp1C	DDB0185020	Macropinocytic pathway [80]	Response to bacterial pathogens [431]
	Tpp1D	DDB0309140	Not known	Not known
	Tpp1E	DDB0304592	Not known	Not known
	Tpp1F	DDB0214912	Golgi complex [165] Endoplasmic reticulum [165] V-ATPase-positive vesicles [165] Extracellular space [77, 78] Macropinocytic pathway [80]	Not known
CLN3	**Cln3**	DDB0233983	Contractile vacuole system [223, 224] Endocytic pathway [223, 224] Golgi complex [78] Macropinocytic pathway [80]	Cell proliferation [223] Cytokinesis [225] Pinocytosis [223] Endo-lysosomal pH [226] Osmoregulation [225] Protein secretion [223, 224] Nitric oxide homeostasis [226] Cell adhesion [224] Timing of early-stage development [224]

				Timing of mid-stage development [223] Timing of late-stage development [223] Spore integrity [225] Response to bacterial pathogens [431]
DNAJC5/CLN4	***DDB_G0290017***	DDB0306688	Macropinocytic pathway [80]	Not known
	Ddj1	DDB0215016	Centrosome [263] Phagosome [262] Macropinocytic pathway [80]	Phagocytosis [262]
CLN5	**Cln5**	DDB0234077	Endoplasmic reticulum [283] Cell cortex [235] Contractile vacuole system [235] Extracellular space [235, 283] Macropinocytic pathway [80]	Autophagy [235] Cell adhesion [235]
MFSD8/CLN7	**Mfsd8**	DDB0307149	Macropinocytic pathway [80]	Not known
CTSD/CLN10	**CtsD**	DDB0215012	Endocytic pathway (e.g., phagosome, lysosome) [352-354] Extracellular space [78] Macropinocytic pathway [80]	Bacterial killing [357] Cell death [359, 360] Timing of early-stage development [354]
PGRN/CLN11	**Grn**	DDB0238428	Not known	Not known
ATP13A2/CLN12	**Kil2**	DDB0237611	Phagosomal membrane [409] Macropinocytic pathway [80]	Bacterial killing [409, 410] Predation on yeast [414]
CTSF/CLN13	**CprA**	DDB0201647	Extracellular space [78]	Osmoregulation [426]
	CprB	DDB0214998	Extracellular space [78]	Phagocytosis [131]
	DDB_G0291191	DDB0252831	Endocytic vesicles [430] Extracellular space [77, 78] Macropinocytic pathway [80]	Response to bacterial pathogens [431]
KCTD7/CLN14	**Kctd9**	DDB0231824	Not known	Not known
	DDB_G0269760	DDB0238663	Not known	Not known
	DDB_G0285861	DDB0346929	Macropinocytic pathway [80]	Not known
	DDB_G0269022	DDB0347398	Not known	Not known

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