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Review

Use of model organisms for the study of neuronal ceroid lipofuscinosis ☆

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ABSTRACT

Neuronal ceroid lipofuscinoses are a group of fatal progressive neurodegenerative diseases predominantly affecting children. Identification of mutations that cause neuronal ceroid lipofuscinosis, and subsequent functional and pathological studies of the affected genes, underpins efforts to investigate disease mechanisms and identify and test potential therapeutic strategies. These functional studies and pre-clinical trials necessitate the use of model organisms in addition to cell and tissue culture models as they enable the study of protein function within a complex organ such as the brain and the testing of therapies on a whole organism. To this end, a large number of disease models and genetic tools have been identified or created in a variety of model organisms. In this review, we will discuss the ethical issues associated with experiments using model organisms, the factors underlying the choice of model organism, the disease models and genetic tools available, and the contributions of those disease models and tools to neuronal ceroid lipofuscinosis research. This article is part of a Special Issue entitled: The Neuronal Ceroid Lipofuscinoses or Batten Disease.

1. Introduction

The study of disease mechanisms and the development and testing of therapeutic strategies for the neuronal ceroid lipofuscinoses (NCLs) presents significant challenges: the use of model organisms is key to achieving these goals. This group of clinically related storage disorders predominantly affects children, causing a progressive neurodegeneration causing dementia, epilepsy, blindness and motor dysfunction leading to premature death. Indeed, although individually rare, the NCLs are the most common cause of childhood neurodegeneration and dementia with an incidence between 1:12,500 and 1:100,000 (depending on the country) [1] and therefore they pose a significant social and economic burden on families, the health service and the state. Details of the disease in humans are discussed in detail in other publications in this special edition. NCL is defined by the progressive accumulation of lysosomal aggregates together with the dysfunction and death of specific populations of neurons within the central nervous system. The identification of distinct NCL disease-causing mutations [2] has enabled studies into protein function, and pathomechanisms, as well as the identification of therapeutic targets and compounds that modulate those targets, and facilitated progress towards the systematic testing of potential therapeutic compounds including the ability to use therapeutic strategies based on gene, protein and stem cell transfer. These new studies have required the use of model organisms, as cell and tissue culture systems cannot replicate the complexity of the central nervous system or indicate how a whole organism may respond to an experimental therapy. Most importantly, experimental colonies can provide sufficient numbers for statistical analysis to support a robust conclusion.

To move towards these goals, it is necessary to identify or generate organisms that model each form of NCL. In addition, expressing human genes in model organisms has enabled identification of their functions and interacting partners. A range of organisms and mutations is now available and more are in development. In this review, in addition to a summary of organisms with mutations in NCL genes, we present the other strains of model organisms available for systematic research, and highlight the most recent and important studies on NCL that employed the use of model organisms. Each animal has its own distinct advantages and limitations for experimental studies. Organisms used to date range from single celled yeast, to invertebrates such as the fruit fly and nematode, through to small vertebrates such as the zebrafish and mouse, and large vertebrates such as dogs, sheep and cattle, each of which has orthologues of at least two CLN genes (reviewed in [3]).

In this review we will discuss each species that has made a significant contribution to NCL research since the last review in this journal [4] and the recent book [5]. As there is no recent data utilising the nematode, this organism is not included. For the organisms presented we summarise their advantages and their use in modelling NCL, understanding NCL biology and experimental therapeutic testing. We

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will also highlight common hypotheses about NCL neuropathogenesis that have arisen from the study of these model organisms.

2. Ethical considerations

Research relating to rare and fatal inherited diseases of children and the development and evaluation of therapeutic approaches for such diseases creates numerous ethical dilemmas for patients, their families, health professionals, researchers and regulatory bodies. In the specific context of animal models for such diseases ethical considerations relate largely to two themes: firstly, the moral status of animals and hence the acceptability of using animals in research that is of only limited or no benefit for the animals themselves, and secondly, the validity of animals as models for human disease. However, it is important to note that the large animal models of the NCLs have been caused by naturally occurring mutations, were diagnosed by veterinarians concerned with the disease in these species, and especially in dogs and cattle the characterisation of the genetic defect resulted in DNA diagnostics that allow effective management via controlled breeding programmes. Thus, research on these models has at least initially been primarily concerned with animal welfare. As with many ethical dilemmas, the co-existence of well informed divergent views, which are based on different philosophical, cultural, social and/or religious frameworks results in a continuum of views in relation to both the moral status of animals [6–8] and the validity of animal models [7,9,10]. Only respectful, considerate and open-minded discussion among all stakeholders can lead to acceptable solutions and the role of researchers in such 'shared moral reflection' [11] is crucial. Researchers who are using animal models for NCL are aware of these issues and reflect and engage publically in the ethical discourse [12–14].

A detailed discussion of the moral status of animals is beyond the scope of this paper, but it should be acknowledged that the moderate utilitarian approach that underpins legislation relating to animal research in many countries considers that animals have some moral status and accepts a balancing of the costs to one moral agent versus benefits to another moral agent. Under this framework the use of animal models can be morally justified with an implicit need to minimise suffering in animals and to prove adequate benefits of research [7,15–17].

Similarly, a more comprehensive review on the debate about the validity of animal models is attempted elsewhere [14]. However, in the context of this paper one might ask which species is the best or most valid model for NCL and how knowledge gained from these models is translated to the human context. Unfortunately, there is no one species that is the best model for all the various biomedical questions we are investigating. A species that is biologically most similar to humans appears often attractive but on technical and ethical grounds may not always be the most practical model [18,19]. The use of multiple animal species as models in NCL research allows for synergistic outcomes and cross-validation of research findings. For example, in relation to preclinical trials for therapeutic approaches for inherited diseases there is an increased awareness that both small and large animals are required [20,21].

Care needs to be taken as to what conclusions for human disease can be drawn from animal models. It needs to be clear that a model is a model and thus a simplification and/or analogue of disease in humans — and disease in humans is complex. This notwithstanding, as discussed in this paper, knowledge gained in NCL animal models has in the past been transferable to the human context and has been essential in advancing our understanding of genetics, biochemistry and pathomechanism and is crucial in the preclinical evaluation of therapeutic approaches. For rare inherited diseases that affect children, such as NCL, clinical trial design is often impacted by important ethical constraints to minimise harm to vulnerable research participants [22], in these situations thorough evaluation of therapeutic interventions by a range of animal models appears to be essential.

3. Large animals

The use of non-laboratory or large animal models for NCL has recently been extensively reviewed [23]. Since the report of 'lipid dystrophic changes' in two English setter dogs [24] an extensive literature has arisen reporting confirmed and suspected naturally occurring cases of NCL in a large variety of species including other dog breeds (Table 2), sheep, cattle, ferrets, cats, horses, goats, pigs, birds, monkeys (Table 1) and mice (Table 3). Identification of NCLs in animals is significant to enable veterinarians to diagnose the disease and provide a prognosis to patient owners as well as to allow researchers to develop diagnostic tests for the management of these disorders at a breed level. More importantly, animals can be indispensable in gaining knowledge of the analogous human diseases. The studies by Nils Koppang of English setters [25,26] affected by the disease were a central stimulus for the first international NCL conference, the 'International Symposium on Human and Animal Models of Ceroid-lipofuscinosis' in 1980 [27]. The ovine CLN6 model quickly followed [28] but it was not until the 1990s that murine models started to become available. More recently, there is increased awareness for the need to utilise large animal models in addition to laboratory animals [20,21,23]. Large animals are more similar to humans in relation to size (including brain size and structure), lifespan (Table 1), physiology and clinical signs of NCL disease (Table 1) and are therefore considered essential in relation to investigations of complex pathomechanisms and preclinical trials for new therapeutic approaches.

Research relating to NCL in large animals reflects these purposes. For many non-laboratory species the literature has described clinical signs and post mortem pathology investigations of individual or familial cases of animals affected by NCL, for some breeds the disease causing gene and mutations have been identified, and experimental populations of sheep and dogs affected by NCL are maintained to increase our understanding of the underlying disease pathomechanism or to evaluate therapeutic approaches (Tables 1 and 2).

3.1. Ovine

Previous literature has described in detail how the ovine experimental NCL populations have increased our knowledge about NCL [23,29–31]. Most influential has been the extensive research on the South Hampshire CLN6 model [28] spanning over three decades of work. Experimental flocks for a genetically different Merino CLN6 model [30,32] and a Borderdale CLN5 model [31,33] have been more recently established. A congenital CLN10/CTSD Swedish Landrace model [29,34] is no longer available as a research population.

3.1.1. Past research in ovine models identified key aspects of disease mechanism, provided leads for therapeutic approaches and insights into genetics

Research in ovine models for NCL has been essential to increase our understanding of the complex disease mechanism and is here only briefly summarised. Early research in the South Hampshire CLN6 model [35–42] did not support previous hypothesis of pathogenesis, i.e. the idea that the disease mechanism was related to dysfunction of the regulation of peroxidation of lipids [43–46] and the hypothesis of a defect in retinoic acid catabolism [47]. The identification and characterisation of subunit c of mitochondrial ATP synthase as the major protein storage component in South Hampshire sheep [36,41,48–52] led subsequently to the important recognition that subunit c of mitochondrial ATP synthase is the main storage component for the majority of NCL variants in humans and animals (Table 1) [5,50]. Furthermore, studies in South Hampshire sheep identified that excitotoxicity was not causative in relation to neurodegeneration as previously suggested

Table 1
Summary of large animal models with confirmed or suggested NCL (dogs not included).

Species/breed(Life span[a])	Gene	Genetic mutation	Molecular defect	Onset of clinical signs	Retinal involvement	Mortality	Clinical signs [cd]				Storage material [cd]				Original References
							VI	BC	MD	S	F	US	SCMAS / SAPs	D	
Sheep (20)															
South Hampshire	CLN6	deletion of exon 1	Lack of protein	7 – 12 m	Yes	25 – 30 m	Yes	Yes	Yes	Yes	Yes	Lamellar	SCMAS	General	[28, 30, 64, 94]
Swedish Landrace	CTSD/CLN 10	G[b]>A	Asp[b]>Asn non-functional protein	at birth	Yes	< 1 m	nd	Yes	Yes	Yes	Yes	GRODs	SAPs	General	[29, 34]
Merino	CLN6	c.184C>T	p.Arg62Cys non-functional protein	7 m	Yes	19 – 27 m	Yes	Yes	Yes	Yes	Yes	Lamellar	SCMAS	General	[30, 32, 95]
Borderdale	CLN5	c.571+1G>A	Splicing variant, truncated protein	10 – 11 m	Yes	24 m	Yes	Yes	nd	nd	Yes	Lamellar	SCMAS	General	[31, 33] D.N. Palmer personal communication
Rambouillet	n.d.			8 m	Yes	24 m	Yes	Yes	Yes	nd	Yes	nd	nd	Neuronal	[96-98]
Cattle (20)															
Devon	CLN5	c.662dupG	p.Arg221GlyfsX6 truncated protein	9 m	Yes	39 m	Yes	Yes	Yes	no	Yes	Lamellar	SCMAS	General	[99-101]
Beefmaster				12 m	nd	18 m	Yes	Yes	nd	Yes	nd	Lamellar	nd	General	[102]
Holstein				nd	Yes	18 m	Yes	nd	nd	nd	Yes	Lamellar	nd	General	[103]
Ferret (12)															
Domestic				> 3 y	Yes	nd	Yes	Yes	Yes	nd	Yes	Lamellar	SCMAS	General	[104] M. France personal communication
Domestic				3 m	nd	4 m	nd	Yes	Yes	nd	Yes	GRODs	SAPs	General	[92]
Cat (34)															
Domestic short-haired				8.5 m	nd	9 m	Yes	nd	Yes	Yes	Yes	Lamellar	SCMAS	Neuronal	[105]
Siamese				< 22 m	nd	23 m	Yes	Yes	Yes	Yes	nd	Lamellar	nd	General	[106]
Domestic short-haired				15 m	Yes	20 m	Yes	Yes	no	Yes	Yes	Lamellar	nd	Neuronal	[107]
Japanese				7 m	nd	11 m	nd	nd	Yes	Yes	Yes	GRODs	nd	General	[108]
Horse (50)															
Aegidienberger				6 m	nd	24 m	Yes	Yes	Yes	nd	Yes	Lamellar	SCMAS	General	[109]
Goat (20)															
Nubian				10 – 18 m	nd	2 – 4 y	nd	Yes	Yes	nd	Yes	Lamellar	nd	Neuronal[e]	[110]
Pig (20)															
Vietnamese pot–bellied				2 y	nd	2.5 y	no	no	Yes	no	Yes	Lamellar GRODs	nd	Neuronal	[111]
Parrot															
Lovebird (12)				< 9 m	nd	9 m	nd	nd	Yes	Yes	Yes	nd	nd	Neuronal	[112]
Monkey															
Cynomolgus monkey (37)				Preclinical at 7 y							Yes	variable	nd	General	[113]
Duck (29)															
Mallet duck				1 y	nd	3 y	Yes	Yes	Yes	Yes	Yes	nd	nd	Neuronal	[93]

Grey Active experimental populations are shaded in grey, nd = not reported, SAPs = sphingolipid activator proteins, SCMAS = subunit C of mitochondrial ATP synthase.
[a]Maximum life span in years (http://www.demogr.mpg.de/cgi-bin/longevityrecords/entry.plx/).
[b]Corresponding to G934 and Asp295 of corresponding human CTSD sequence.
[c]VI = visual impairment, BC = behavioural changes, MD = motor deficits, S = seizures.
[d]F = fluorescent, US = ultrastructure, D = distribution.
[e]Only central nervous system investigated.

[53] and that instead observed changes in interneuron populations followed pathogenesis [54,55].

The importance of the role of neuroinflammation in NCL prior to neurodegeneration was first identified in time course studies on disease progression in South Hampshire sheep [56,57] and has led to the investigation of anti-inflammatory drugs as new therapeutic approaches in affected South Hampshire sheep (D. N. Palmer, personal communication) and other species.

Previously, affected South Hampshire sheep have been useful in the evaluation of bone marrow transplantation [58] and more recent work in relation to gene therapy is described below.

The identification of CLN6 and CLN5 as causative genes in the South Hampshire, Merino and Borderdale sheep supported previous findings that these genes caused disease in humans. More importantly, the identification of CTSD as the disease causing gene in White Swedish Landrace sheep [29] was the first report of an NCL caused by a mutation in a major

Table 2
Summary of canine models with confirmed or suggested NCL.

Breed	Gene	Genetic mutation	Molecular defect	Onset of clinical signs	Retinal involvement	Mortality	Clinical signs [a]					Storage material [b]			Original References
							VI	BC	MD	S	F	US	SCMAS / SAPs	D	
Confirmed NCL															
Miniature dachshund	PPT1/CLN1	c.736_737insC	Frameshift after Gly245 with stop codon at position 276	9 mo	Yes	nd	Yes	Yes	Yes	No	Yes	GRODs	nd	Neuronal	[114]
Longhaired Dachshund	TPP1/CLN2	c.325delC	Frame shift after amino acid 107 (exon 4) with stop codon at position 114	7 - 9 mo	Yes	12 mo	Yes	Yes	Yes	Yes	Yes	Lamellar	nd	Neuronal	[85, 88, 115, 116]
Border collie	CLN5	c.619C>T	Truncated protein (stop codon Q206X)	16 - 23 mo	Yes	28 mo	Yes	Yes	Yes	Yes	Yes	Lamellar	nd	Neuronal	[117-121]
Australian shepherd	CLN6	c.829T>C	Missense Trp277Arg	1 - 2 y	Yes	nd	Yes	Yes	Yes	nd	Yes	Lamellar	nd	Neuronal	[122, 123]
English setter	CLN8	c.491T>C	Missense L164P	1 - 2 y	Yes	2 y	Yes	Yes	Yes	Yes	Yes	Lamellar	SCMAS	Neuronal	[124-142]
American bulldog	CTSD/CLN10	c.?G>A	Missense Met199Ile	1 - 3 y	no	7 y	no	Yes	Yes	no	Yes	Round uniformly staining inclusions embedded within granular matrixes	nd	Neuronal	[143-145]
Tibetan terrier	ATP13A2/CLN12	1620delG	Skipping of exon 16 leading to shortened protein	5 - 7 y	Yes	nd	Yes	Yes	Yes	Yes	Yes	Lamellar; granular contents	nd	Neuronal	[78, 79, 146-149]
American Staffordshire (Pit Bull) Terrier	ARSG	296G>A	Missense Arg299His	3 - 5 y	no	nd	no	no	Yes	no	nd	Lamellar	nd	Neuronal	[83, 150]
Australian shepherd (not CLN6)	nd	nd	nd	15 mo	Yes	nd	Yes	Yes	Yes	nd	Yes	Lamellar	nd	Neuronal	[122]
Polish owczarek Nizinny/Polish Lowland sheepdog	nd	nd	nd	6m - 4y	Yes	8 y	Yes	Yes	Yes	no	Yes	GRODs	SAPs	General	[137, 151-153]
Miniature schnauzer	nd	nd	nd	2 y	Yes	nd	Yes	Yes	Yes	no	Yes	GRODs	SAPs	Neuronal	[154-157]
Chihuahua	nd	nd	nd	16 - 18 mo	Yes	24 mo	Yes	Yes	Yes	no	Yes	Lamellar	nd	General	[158, 159]
Cocker spaniel	nd	nd	nd	18 mo	Yes	6 y	Yes	Yes	Yes	Yes	Yes	Lamellar	nd	Neuronal and General	[160-162]
Dalmatian	nd	nd	nd	6 mo	Yes	6 yr	Yes	Yes	Yes	Yes	Yes	Lamellar	nd	General	[163, 164]
Japanese retriever	nd	nd	nd	3 y	nd	nd	nd	nd	nd	Yes	Yes	nd	nd	General	[165]
Welsh corgi	nd	nd	nd	6 - 8 yr	Yes	nd	Yes	Yes	nd	Yes	nd	nd	nd	nd	[166]
Labrador retriever	nd	nd	nd	7 y	no	nd	no	no	Yes	Yes	nd	Lamellar	nd	Neuronal	[167]
Australian cattle dog	nd	nd	nd	1 y	Yes		Yes	no	Yes	no					[168, 169]
Saluki	nd	nd	nd	1 y	no	2 y	no	Yes	Yes	no	Yes	Lamellar	nd	Neuronal	[170]

Grey: Active experimental populations are shaded in grey, nd = not reported, SAPs = sphingolipid activator proteins, SCMAS = subunit C of mitochondrial ATP synthase.
[a] VI = visual impairment, BC = behavioural changes, MD = motor deficits, S = seizures.
[b] F = fluorescent, US = ultrastructure, D = distribution.
[c] Only central nervous system investigated.

lysosomal protease and the first study of an NCL animal model that has led to the identification of a new candidate gene for a human NCL [59].

Finally, CLN5 and CLN6 sheep models have allowed for the systematic generation of research resources such as RNA, DNA and tissue samples as well as neuronal cell cultures that have, in addition to similar material from laboratory animals, assisted in basic research relating to normal and abnormal function of NCL genes and proteins (e.g. [60–63]).

3.1.2. Current research

Since the last review [23] research relating to experimental NCL populations in South Hampshire, Merino and Borderdale sheep was

recently presented at the 13th International Conference on Neuronal Ceroid Lipofuscinoses (http://www.ncl2012.org/). Firstly, a large deletion that includes exon 1 of the CLN6 gene has been identified as the disease causing mutation in the South Hampshire model [64], confirming previous genetic research that proposed South Hampshire sheep as a model for CLN6 disease [30]. Secondly, research using this South Hampshire model in relation to pathogenesis explored the role of cytokines in neuroinflammation [65] and the role of alterations in metal homeostasis and cellular signalling pathways in the brain of affected sheep [66]. Furthermore, primary sheep neuronal cells were used in a study of early changes to lysosomes and the endoplasmic reticulum with the aim to develop a better understanding of early pathology which could assist in the development of in vitro tests for the evaluation of current gene therapy trials in these sheep [67].

Finally, progress has been made in relation to using sheep models to develop or evaluate therapeutic approaches. Longitudinal biomarkers of disease progression, including behaviour studies, computer tomography and magnetic resonance imaging, are developed for all three experimental sheep populations [68–70]. The potential role of neuronal progenitor cells and neurogenesis as natural attempts of brain repair is investigated. Evidence of increased neurogenesis in the subventricular zone and possible clusters of newly generated neurons in the cortex was found in the CLN6 South Hampshire sheep, in the CLN5 Borderdale model and in human CLN6 patients but these cell clusters in the cortex were absent in the CLN6 mouse model [71,72]. This finding strengthens our argument that the use of a diversity of animal models and especially large animal models with brains and pathology more similar to humans is important and further research is required to identify how this phenomenon can be useful for therapeutic approaches. Chimeric sheep generated from CLN6-affected and normal embryos have been investigated to explore the ability of in vivo intercellular correction for NCL caused by membrane proteins [73]. Initial promising findings suggest that intercellular correction is not impossible, as previously believed, thus creating new opportunities for therapeutic interventions such as gene therapy or stem cell therapy. Preliminary findings in a study that evaluated the calpain inhibitor (CAT0811) as an anti-neurodegenerative therapeutic agent in CLN5 Borderdale sheep were presented [69]. Most importantly, previous investigations into lentiviral mediated gene transfer with the aim to develop gene therapy for NCL [74] have progressed. Further work on the generation and testing of lentiviral vectors for CLN5 and CLN6 and tests on transgene expression and function in vivo in a large human-like brain and in vitro were presented [70,75].

3.1.3. Summary

Extensive and systematic research in four well characterised ovine models of NCL for more than three decades provided key insights in the disease mechanisms and informed development and evaluation of new therapeutic approaches. Most importantly, early misconceptions with regard to disease mechanism could be rejected based on ovine research and the findings that subunit c of mitochondrial ATP synthase is the major protein storage component in most variants of NCL and that neuroinflammation plays an important role in pathogenesis shifted our understanding of the disease mechanisms and informed the development of new therapeutic approaches. A new candidate gene for NCL – CTSD – was proposed and confirmed as disease causing in humans. In the future, the existence of these well characterised and easy to maintain ovine research flocks will be vital to further unravel the poorly understood disease mechanism and to continue the development and evaluation of therapeutic approaches.

3.2. Canine

There is a wealth of literature describing the clinical and pathological consequences of mutations in NCL genes in various dog breeds (see the references in Table 2 and [76]), providing important criteria for diagnosis and for the monitoring of experimental therapy testing. To date, studies on NCL in dogs have significantly advanced the repertoire of genes that are known to cause NCL when mutated, provided mechanistic insights and enabled the testing of experimental therapies in a relatively large brain.

3.2.1. Identification of candidate NCL genes also provides mechanistic insights

Diagnostic tests are available for many dog breeds and are being used widely to reduce breeding of carrier dogs. Since the last review and the first high quality build of the canine genome in July 2004 [77], the causative mutations in 8 breeds have been found (Miniature Dachshund, Longhaired Dachshund, Border Collie, Australian Shepherd, English Setter, American Bulldog, Tibetan Terrier and American Staffordshire Terrier; Table 2). Most significantly, one naturally occurring model has demonstrated that novel mutations in genes already known to underlie other neurodegenerative diseases also cause NCL. The Tibetan Terrier has a mutation in *ATP13A2* [78,79], and mutations in the same gene are also found in a rare form of Parkinson's disease called Kufor–Rakeb syndrome. As it has been demonstrated that ATP13A2 is protective against manganese induced toxicity in mammalian cells and yeast [80,81], this already points to a putative disease mechanism not previously linked to NCL that requires further investigation. The identification of the mutation in *ATP13A2* in Tibetan Terriers with NCL [78,79] supported the demonstration that a mutation in ATP13A2 causes CLN12 disease in an NCL-affected family in Belgium [82]. Similarly, the identification of mutations in the canine *arylsulfatase G* (*ARSG*) gene as a cause of NCL in American Staffordshire Terriers [83] (Fig. 1) provides a novel candidate gene for sequencing in NCL patients for whom the underlying mutation is not known. At least eleven breeds of dogs with NCL still harbour unidentified mutations but these are either old or isolated cases and until new cases are found the underlying mutation is unlikely to be identified [76]. However, these breeds still remain a potential source of novel NCL genes that will provide candidates for mutations in humans.

Although examination of NCL in dogs has provided important mechanistic insights into the disease in the past (reviewed in [76]), the identification and generation of mouse models seem to have resulted in a reduction in the use of dogs for mechanistic studies.

3.2.2. Development of experimental therapies

The canine NCL models are increasingly being exploited for the testing of experimental therapies because of several advantages: to study the effect of the therapy on a larger brain that is more similar to the human brain than the rodent brain is, to monitor the outcome of the therapy over a protracted period, and to test the therapy in a model whose clinical progression resembles that of a human more closely. Experimental colonies are necessary for the testing of experimental therapies, and colonies of Longhaired Dachshund (CLN2 mutation) and English Setters (CLN8 mutations) exist. Although much of the work is still unpublished, it was revealed at the 13th International Conference on Neuronal Ceroid Lipofuscinosis (www.ncl2012.org) that these dogs are being carefully examined for biomarkers and quantitative clinical assays for use when assessing the efficacy of experimental therapies [84,85] and the Longhaired Dachshund has been employed to test a variety of methods for delivery of TPP1 to the canine brain including intrathecal and intracerebral ventricular administration of recombinant protein [86–88], and AAV-mediated transduction of brain ependyma [89]. Importantly, the use of dogs allows the delivery device to be tested for suitability, as well as indicating the spread and relative expression levels of TPP1, and the efficacy of the treatment. Based on efficacy in the canine TPP1 mutant, together with toxicology and pharmacokinetic data from a variety of species including non-human primates, Biomarin announced that it is planning a clinical trial for rhTPP1 treatment during 2013. However, studies in mice on the use of combinatorial treatments to treat CLN1 disease have shown synergistic effects [90,91], suggesting

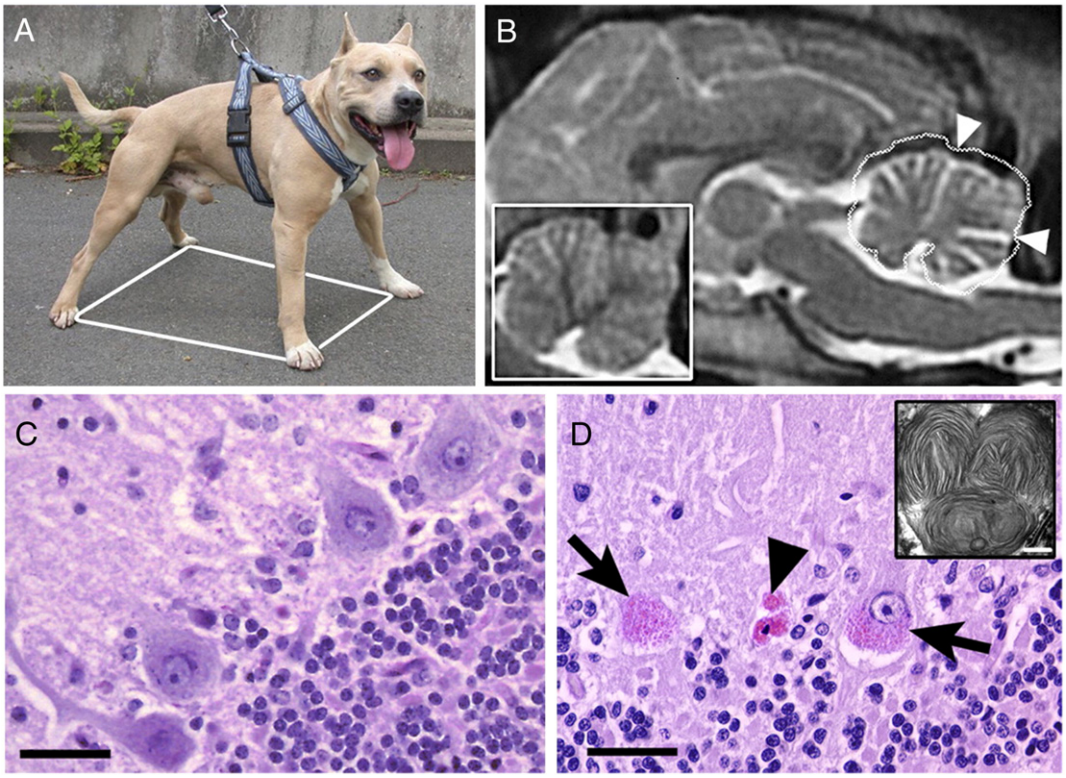

Fig. 1. Clinical and histopathological features of canine arylsulfatase G deficiency. (A) The wide-based stance (white polygon) of a 5-year-old affected American Staffordshire Terrier (AST) illustrates loss of motor coordination. (B) Sagittal 2-T weighted MRI of the brain from a 6-year-old AST through the cerebellum. (Inset) A similar image at the same scale of the cerebellum from an age-matched healthy AST, the outline of which has been projected on the cerebellum of the main image (white dotted line). A reduction of grey matter is demonstrated by the enlarged sulci (arrowheads). (C and D) Transverse sections of the cerebellum from healthy dogs (C) and affected dogs (D) stained with PAS reagent and counterstained in Mayer's hematoxylin solution. (C) In a normal cerebellum, the large PAS-negative Purkinje neurons lie between the molecular (top left) and granular (bottom right) layers. (D) In the cerebellum from an affected dog, massive Purkinje cell loss results in a blurry line. The remaining Purkinje neurons (arrows) or neuron remnants (arrowheads) accumulate perinuclear PAS-positive granular material. (Inset) Affected Purkinje neurons imaged on transmission electron microscopy show accumulated lysosomal material composed of concentric straight or curved profiles with alternating clear and dense bands. (Scale bars: 50 μm for the sections; 250 nm for the inset.)
Reproduced with permission [83].

that combinatorial treatments such as rhTPP1 and anti-inflammatory compounds should also be trialled in the canine model of CLN2 disease to see if efficacy can be further improved.

3.2.3. Summary

Canine models of NCL have resulted in the identification of new candidate genes for human NCL, provided mechanistic insight into NCL through the identification of novel disease genes and pathological examination, and enabled testing of experimental therapies. In the future, canine NCL models will continue to provide new candidate genes until the mutations have been identified in all of those breeds for which there is suitable tissue available. This will lead to a specific diagnosis for more patients and further insights into disease mechanisms. The use of the canine laboratory populations for testing of experimental therapies will continue to be extremely important, and the formation of further colonies with mutations in different genes will provide an important resource for the testing of experimental therapies for more forms of NCL.

3.3. Other large animals

Many other large animal models for NCL exist and are summarised in Table 1 and in the previous review [76]. Two recent reports of naturally occurring NCLs in non-laboratory species include a new variant of NCL in a ferret [92] and a duck that was suspected to have NCL [93], and key findings are included in Table 1.

4. Mouse

Mouse models are an excellent genetic tool to gain insight into the pathogenesis of neurodegenerative diseases. In the last decade, understanding of the molecular mechanisms underlying NCL has been enormously improved due to studies on mice recapitulating the clinical features of this devastating disorder. NCL mouse models have also proven useful for evaluating the benefits of potential therapeutic treatments. It is, however, important to bear in mind that the data obtained needs to be carefully transferred to humans as rodents exhibit less pronounced neuroanatomical structures and a relatively simple behaviour pattern.

The existing NCL mouse models originate either from naturally occurring mutagenesis or have been developed through biotechnological modifications targeting a specific gene or gene locus to mimic accurately human genotypic defects (Table 3, genetically accurate models are highlighted in bold). To facilitate direct comparison, there has been a concerted effort to establish the same strain backgrounds, C57BL/6, across the different mouse models. As a result, several mouse strains are available now that model the major NCL forms of childhood (Table 3). Collectively, they all show severe brain atrophy and autofluorescent storage material accumulation. Other mice have been discovered that display an NCL-like phenotype but as yet these do not correlate with any known human NCL.

It is evident that research has focused largely on structural or functional changes in the brain of each mouse model. Therapeutic achievements have particularly been made in NCL mice expressing mutant soluble proteins; whereas, the development of possible

Table 3
Summary of mouse models with confirmed or suggested NCL.

Human NCL	Mouse model	Genetic mutation	Molecular defect	Onset of neurological signs	Retinal involvement	Synaptic alteration	Mortality	Clinical Signs [c]				Storage material [d]			Original Reference	Therapeutic intervention in mice
								VI	BC	MD	S	F	US	SCMAS/ SAPs		
CLN10 disease, congenital	**Ctsd^{-} knockout**	Ex. 4 neo cassette insertion	No enzyme activity	3 wk	+	+	26 ± 1d	+	nd	nd	+	+	GROD	SCMAS	[173]	AAV gene therapy [259]
CLN1 disease, infantile	Ppt1^{-} knockout	Ex. 9 neo cassette insertion	No enzyme activity	< 2 m	+	+	< 5 m	+	+	+	+	+	GROD	SCMAS	[180]	Enzyme replacement therapy [255] AAV gene therapy [264, 265] AAV gene therapy & bone marrow transplantation [266] Stem cell transplantation [267]
	Ppt1$^{\Delta ex4}$ knockout	Ex. 4 deletion	No enzyme activity	< 4 m	+	nd	6.5 m	+	nd	+	+	+	nd	SAPs	[181]	nd
CLN2 disease, late-infantile	Tpp1 neo^{ins} Arg446His knockout	Ex. 11 missense mutation & In. 11 neo cassette insertion	No enzyme activity	< 9 wk	nd	nd	nd	nd	+	+	+	+	nd	nd	[193]	Enzyme replacement therapy [194, 257] AAV gene therapy [260-262]
CLN5 disease, variant late-infantile	Cln5$^{-/-}$ knockout	Ex. 3 neo cassette insertion	Deleted mRNA	13 wk	< 13 wk	+	nd	+	nd	nd	nd	+	CLP, FPP	nd	[195]	nd
CLN6 disease, variant late-infantile	**Cln6^{nclf} spontaneous**	Single base pair insertion	Frameshift after Pro102	< 8 m	+	+	12 m	nd	nd	+	nd	+	CCLP, FPP, RLP	SCMAS	[199]	nd
CLN8 disease, variant late infantile	**Cln8^{mnd} spontaneous**	Single base pair insertion	Frameshift after Val89	< 2 - 3 m	+	+	10 - 12 m	+	+	+	+ [b]	+	CLP	SCMAS	[203]	AMPA receptor antagonist [272] Carnitine [271, 273] β2-adrenoceptor agonist [274]
CLN3 disease, juvenile	Cln3$^{\Delta ex7/8}$ neo knockout	Ex. 7-8 neo cassette insertion	Deleted mRNA, splicing	< 14 wk	Reduced cell density in retina	nd	nd	+	+	nd	nd	+	CCLP, FPP, RLP	nd	[220]	nd
	Cln3$^{\Delta ex1-6}$ knockout	Ex. 1-6 neo cassette insertion	Deleted mRNA, absent protein	< 16 m	Optic nerve degeneration <6m [198]	+	20 m	nd	nd	+	+ [a]	+	RLP, FPP	SCMAS	[221]	AMPA receptor antagonist [269] NMDA receptor antagonist [270] Immunosuppression [275]
	Cln3$^{\Delta ex7/8}$ knock-in	Ex. 7-8 deletion	Deleted mRNA, splicing variant	10 - 12 m	+	+	< 7 m	+	+	+	nd	+	FPP	SCMAS	[222]	nd
	Cln3$^{lacZ/lacZ}$ knock-in	LacZ insertion & ex 2-8 and most of ex. 1 deletion	β-gal expression, absent CLN3 protein	< 2 m	nd	nd	nd	nd	+	+	+ [b]	+	nd	nd	[223]	nd

nd = not reported, SAPs = sphingolipid activator proteins, SCMAS = subunit C of mitochondrial ATP synthase, CLP = curvilinear profile, FPP = fingerprint profile, RLP = rectilinear profile.
[a] Increased seizure-induction latency in immature animals aged 35–42 days [285].
[b] Enhanced susceptibility to drug induced seizures.
[c] VI = visual impairment, BC = behavioural changes, MD = motor deficits, S = seizures.
[d] F = fluorescent, US = ultrastructure.

treatments for NCL forms affecting transmembrane proteins remains challenging. The present work summarises the pathologic characteristics and new therapeutic insights on the current NCL mouse models, therewith providing an update on the last reviews in this journal in 2006 and 2009 [171,172]. Due to the extensive data, the animal models are grouped by disease to indicate conserved and variable phenotypic features. Moreover, candidate NCL mouse models are highlighted and recent therapeutic interventions are briefly presented.

4.1. Mouse models of NCL

The cathepsin D (Ctsd) knockout mouse that carries a mutation in exon 4 of the *Ctsd* gene similar to humans represents an animal model for congenital CLN10 disease. In line with the human pathogenesis, these mice exhibit an aggressive phenotype with progressive neurological symptoms, acute accumulation of autofluorescent storage material and defects in visceral organs. Death usually occurs at the age of 26 ± 1 days [173,174]. Brain areas affected early on are the somatosensory cortex and related thalamic nuclei with profound microglia activation, astrocytosis, axonal degeneration and neuronal death [175]. The cause of the cell death is not known; however, it was suggested that glial cell activation, oxidative stress and autophagy might play a role [174,176,177]. The removal of the pro-apoptotic protein Bax did not significantly decrease neuronal cell loss supporting further the involvement of non-apoptotic death mechanisms in the neuronal degeneration of *Ctsd*$^{-/-}$ mice [178].

Interestingly, morphological and functional alterations of the presynapse were found before the onset of clinical signs consistent

with recent findings showing a reduced frequency of mEPSCs (miniature excitatory postsynaptic currents) at early stages of the disease in *Ctsd*$^{-/-}$ mice [175,179]. The synapse may, therefore, present a potential target for early therapeutic intervention.

Two infantile CLN1 disease mouse models exist that lack Ppt1 (palmitoyl protein thioesterase-1) enzyme activity through the targeted disruption of exon 9 (*Ppt1*$^{-/-}$ knockout) or complete deletion of exon 4 (*Ppt1*$^{\Delta ex4}$ knockout) in the *Ppt1* gene [180,181]. Phenotypically, the two models are similar in that both present with vision loss, spontaneous seizures and motor abnormalities; the progression, however, is slightly faster in *Ppt1*$^{\Delta ex4}$ knockout mice with death at an age as early as 5 months [180,181]. A regional selectivity is apparent in the series of pathogenic events in these mice. Before the onset of neurological signs astrocytosis commences in thalamic nuclei and cortical laminae, spreads extensively and is followed by GABAergic cell loss and microglia activation in corresponding (sub)cortical regions [182,183]. A similar progressive pattern is seen in the cerebellum of *Ppt1* deficient mice [181,184].

In line with findings in *Ctsd*$^{-/-}$ mice, a synaptic pathology was described in these mouse models for infantile CLN1 disease including a decreased frequency of miniature synaptic currents, reduction in synaptic vesicle pool size, defects in synaptic vesicle recycling and altered expression of synaptic protein marker [185–187]. Recently, the function of glutamate receptors was reported to be altered in *Ppt1* deficient neurons [188]. Gene expression profiles indicated impairment in neuronal development, calcium homeostasis, lipid metabolism and trafficking [189]. However, the mechanisms that mediate the ultimate neuronal loss in these mice remain elusive. Oxidative and ER (endoplasmic reticulum) stress as well as UPR (unfolded protein response) and RAGE (receptor for advanced glycation end products) induction were suggested to play a role in caspase activation and subsequent apoptosis [190–192].

The *Tpp1*$^{-/-}$ mouse model mirroring late infantile CLN2 disease was created by a *Cln2*-specific missense mutation (equivalent to p. Arg447His) and a large intronic insertion resulting in the abolishment of Tpp1 (tripeptidyl peptidase 1) enzyme activity. The mice are characterised by a strongly progressive phenotype with clinical features apparent from 7 weeks of age onwards including tremors, spontaneous seizures and rapid loss of motor function [193]. Prominent neurodegeneration and glial activation were observed in the thalamocortical system and the cerebellum accompanied by widespread reactive astrocytosis at disease end stage [193,194]. Vision loss, a significant symptom of human late infantile CLN2 disease, was not reported [193].

The insertion of a neomycin cassette into the exon 3 of the murine *Cln5* gene results in a truncated protein transcript that models variant late-infantile CLN5 disease. These mice display, similar to patients, a relatively mild phenotype without severe brain atrophy or prominent motor abnormalities. By 5 months of age, a progressive visual decline is evident leading to complete loss of vision [195].

In *Cln5*$^{-/-}$ mice microglia activation, astrocytosis and defective myelination are visible from 3 months of age onwards accompanied by an altered expression level of genes involved in immune response, myelin integrity and neuronal degeneration [195–197]. Interestingly, late neuronal loss is reported to become apparent in the cortex before its onset in thalamocortical regions, which is opposite to the order in other NCL mouse models [175,183,197,198]. In line with the clinical features, neurodegeneration is most pronounced in the visual system [197]. In particular, GABAergic interneurons were found to undergo cell death [195]. Immunohistochemistry studies on 12 month old *Cln5*$^{-/-}$ mice revealed alterations in the distribution and level of pre-synaptic proteins in the thalamocortical system and the cortex [197]. Moreover, a dysregulation in cellular metabolism and lipid transport was described in these mice [196].

The *Cln6*^{nclf} mouse is a naturally occurring mutant mouse that bears a frame shift in exon 4 of the *Cln6* gene causing a short-lived truncated CLN6 protein [199,200]. These mice develop progressive retinal atrophy by 4 months of age and rear limb paresis by the age of 8 months. Autofluorescent inclusions are visible in the murine brain and spinal cord early on and spread rapidly leading to widespread accumulation of storage material by 6 months of age. Reactive gliosis, myelin sheath degeneration and localised astrocytosis also become evident [199,201]. Death occurs by 12 months of age [199].

Consistent with other NCL mouse models, evidence for synaptic pathology was provided in the CLN6 mouse model. The expression level of synaptic proteins was reported to change first in the thalamus and later in the cortex, which may indicate a regional selectivity [187]. A decrease in GABA and an increase in glutamate and glutamine were detected in the cortex; in the cerebellum these changes were less pronounced [201]. ER stress and UPR activation could not be associated with neuronal loss. It was suggested, rather, that an up-regulation or disruption of the autophagy-lysosome degradation pathway might be involved in the neurodegeneration of *CLN6*^{nclf} mice, similar to findings in *Ctsd* deficient and *Cln3*$^{\Delta ex7/8}$ mice [177,201,202].

Another naturally occurring mouse model is the *Cln8*^{mnd} mouse harbouring a premature stop codon in the *Cln8* gene that is the counterpart of human variant late infantile CLN8 disease [203]. The mice represent a very similar, yet more aggressive, neurological phenotype to *CLN6*^{nclf} mice [199]. In *Cln8*^{mnd} mice on a C57BL/6 background, progressive retinal atrophy and spastic limb paresis commence at an age as early as 5 weeks and 6 months, respectively. Premature death occurs by 12 months of age [203,204]. It should be noted that mutant mice on an AKR background show earlier and more progressive clinical signs [205].

Before motor deficits become apparent, *Cln8*^{mnd} mice display early-onset microglia activation, astrocytosis, up-regulation of pro-inflammatory cytokines and ER stress in the brain or spinal cord [206–210]. Loss of retinal cells, mostly photoreceptor cells, is found from P20 onwards and can be linked to oxidative stress [211–213]. Concurrent with the beginning of gait abnormalities, neuronal loss is detected in the spinal cord and hippocampus; slightly later neurodegeneration occurs in the cortex [206,214]. In agreement with the pathology of other NCL mice, the murine *Cln8*^{mnd} model shows a loss of GABAergic interneurons in the CNS [214]. Synaptic pathology is described in the spinal cord and CNS including an altered expression of glutamate receptors, increased glutamate levels and neuronal hyperexcitability [209,215–217]. Regional selectivity, a frequently reported characteristic in NCL mouse models, is demonstrated for microglia activation and neurodegeneration with onset in the somatosensory thalamocortical pathway at early disease stages [207,208].

Moreover, *Cln8*^{mnd} mice reveal behavioural abnormalities, namely enhanced activity, aggression, poor memory and associative learning abilities [218,219].

There are four genetically different mouse models for juvenile CLN3 disease, the most common NCL type in humans. Two knockout mice were generated by the insertion of a neomycin cassette into the *Cln3* gene to replace exons 7–8 (*Cln3*$^{ex7/8neo}$) [220] or exons 1–6 (*Cln3*$^{\Delta ex1-6}$) [221]. A knock-in mouse was developed using the *cre-lox* technology to excise exons 7 and 8 of *Cln3* (*Cln3*$^{\Delta ex7/8}$) in order to mimic the 1 kb deletion in *Cln3*, the most common disease-causing mutation [222]. Lastly, a knock-in reporter mouse was designed by replacing most of exon 1 and all of exons 2–8 with the exogenous reporter gene *LacZ* (*Cln3*$^{lacZ/lacZ}$); while, the endogenous promoter sequence was not altered [223]. Thus, this last mouse model allows the study of *Cln3* expression and circumvents the challenges of using antibodies which has proven difficult due to low endogenous *Cln3* expression [223].

Despite, the genetically engineered disruption of the gene sequence, it is debated whether partial *Cln3* activity may be retained in the knockout mouse model [224]. Variant mRNA transcripts of *Cln3* were detected in *Cln3*$^{\Delta ex7/8}$ mice, as in patient samples [222,224], and the phenotype of cells from patients worsened when CLN3 activity was further depleted using RNA inhibition. In further support of

residual enzyme activity, *Cln3* mutant mice mirror relatively late onset and mild progression of human symptoms such as visual decline, motor dysfunctions, memory deficits, resting tremor and decreased overall activity [219–223,225,226]. It is of note, however, that retinal degeneration was pronounced in $Cln3^{\Delta ex7/8}$ mice bred on a mixed 129/Sv/CD1 background and that the CD1 mouse strain is linked to albinism [222].

At early disease stages the $Cln3^{\Delta ex1-6}$ mouse model exhibits a slowly progressive loss of neurons in the striatum, cerebellum and thalamic nuclei, and sparse atrophy was found in the cortex [198,221,227–229]. $Cln3^{\Delta ex7/8}$ mice show selective loss of thalamic neurons, somatosensory relay neurons and target neurons within the somatosensory cortex [229]. Widespread cell death of GABAergic interneurons was reported at the age of 14 months in $Cln3^{\Delta ex1-6}$ mice [230]. Interestingly, retinal cells appeared to be relatively preserved. Degeneration of visual relay neurons in the LGNd (dorsal lateral geniculate nuclei), reduced conduction velocity in the optic nerve and onset of optic nerve degeneration were followed by minimal retinal cell loss in $Cln3^{\Delta ex1-6}$ mice [198,231]. The $Cln3^{\Delta ex7/8}$ mouse model displays a moderate visual phenotype including inner retinal cell loss, reduced inner retinal cell function and altered pupillary light reflexes [116,225].

The mechanism underlying neuronal degeneration in juvenile CLN3 disease is not known. Prior to neuronal death in the striatum, a reduced level and catabolism of dopamine, potentially causing the accumulation of reactive oxidative dopamine forms, was evident in $Cln3^{\Delta ex1-6}$ mice. An altered dopamine catabolism may cause the accumulation of reactive oxidative dopamine forms [227]. An increase in oxidative proteins was also detected in the cerebellum, thalamus and primary cortex of these mice further supporting the involvement of oxidative stress in the neurodegeneration of juvenile NCL [232]. Studies with neuronal cell cultures derived from $Cln3^{\Delta ex7/8}$ and $Cln3^{\Delta ex1-6}$ mice provided evidence that dysregulated autophagy, membrane or vesicle trafficking and mitochondrial function may play a role in neuronal loss [202,233,234].

In comparison to other NCL mouse models, *Cln3* mutant mice show an atypical neuroimmune response. Subtle activation of neuroglia was detected early on in $Cln3^{\Delta ex1-6}$ and $Cln3^{\Delta ex7/8}$ mice; however, astrocytotic proliferation and macrophage formation was not observed [229,230]. Increased levels of α-fetoprotein (that may act as an autoantigen) and circulating autoantibodies to glutamic acid decarboxylase (GAD65) were found, supporting the notion of an autoimmune component to the pathogenesis in juvenile CLN3 disease [235,236]. The presence of GAD65 was associated with elevated levels of presynaptic glutamate, consistent with reported changes in the glutamate/glutamine cycling of *Cln3* deficient mice [236,237]. Other synaptic alterations include reduced levels of GABA and an age-dependent sensitivity to AMPA and NMDA receptor mediated excitotoxicity [237,238].

4.2. Candidate NCL mouse models

Despite advanced gene sequencing technologies, there are variant and adult-onset human NCL cases without known disease-causing genetic defects. Mouse models have been discovered that exhibit phenotypes resembling distinctive NCL features and may represent accurate models for human NCL forms. Candidate NCL mouse models include animals harbouring null mutations in the genes for *Cathepsin F* (*Ctsf*), *Ppt2* (*Palmitoyl protein thioesterase 2*) and for two chloride channels (*Clcn-7*, *Clcn-6*). To date, none of the models has been linked directly to human NCL.

Ctsf is a ubiquitously expressed lysosomal cysteine protease involved in lipoprotein degradation and antigen processing [239–241]. Mice lacking Ctsf show slow progressive widespread accumulation of autofluorescent storage material in the CNS, motor abnormalities and seizures [242] — common NCL characteristics. Intracellular inclusions and motor coordination deficits become apparent at 6 weeks and 12 months of age, respectively, which may point towards a murine model for late onset NCL. However, the adult-onset NCL diseases tested so far did not bear mutations in the *Ctsf* gene [242].

The gene *Ppt2* is another candidate for late onset NCL and encodes a lysosomal thioesterase homologous to the protein Ppt1. *Ppt2* null mutant mice ($Ppt2^{-/-}$) display an NCL-like phenotype including autofluorescent storage material in brain, neuronal cell death and motor defects with an onset later than *Ppt1* deficient mice [180,243]. Unlike the majority of NCL mouse models, however, visceral features affecting pancreas, bone marrow and spleen were observed in $Ppt2^{-/-}$ animals [243].

Mice carrying mutations in the *Clcn-7* and *Clcn-6* genes of the voltage-gated chloride channel family CLC have been proposed as putative NCL mouse models [244,245]. CLCs reside in the plasma membrane and intracellular organelles to exert functions like the regulation of chloride levels and the acidification of vesicles. The disruption of *Clcn* genes leads to a variety of disorders with osteopetrotic, hepatic and neurodegenerative manifestations [246,247]. Mice lacking *Clcn-7* and *Clcn-6* reveal pathological features of NCL disease, however, features are also present that are not found in human NCL patients.

A *Clcn-7* mutant mouse ($Clcn\text{-}7^{-/-}$) designed to abolish protein expression completely was reported to develop severe osteopetrosis, widespread neuronal and retinal degeneration, autofluorescent intralysosomal inclusions and premature death at 7 weeks of age [244,248]. *Clcn-7* deficient mice exhibit localised astrocytosis and microglial activation within the somatosensory thalamocortical system, loss of thalamic relay neurons and cortical interneurons, features that are evident in multiple forms of NCL [175,183,197,249]. A spontaneous mutation in Osmt1, a functionally important β-subunit of Clcn7, leads to a phenotype closely resembling $Clcn\text{-}7^{-/-}$ mice [249,250]. Recently, another *Clcn-7* mutant mouse model was generated bearing an alternate *Clcn-7* transcript. These mice present with early onset and rapidly progressive neurodegeneration similar to the complete loss of *Clcn-7*; interestingly, osteopetrosis is absent. The lack of osteopetrosis results from the expression of alternative splicing transcripts in bones indicating that neuronal and retinal degeneration may not originate from osteopetrotic compression of the brain [251]. Based on these findings *Clcn-7* mutant mice may act as a model for human congenital NCL disease [249,251]. In contrast, the disruption of Clcn-6, a channel exclusively expressed in neurons, is debated to give rise to a mouse model for milder forms of human NCL. $Clcn\text{-}6^{-/-}$ mice have a normal life span with slowly progressing neurodegeneration, distinct accumulation of storage material and mild behavioural abnormalities [245]. The mice, however, do not show evidence of localised thalamic glial response, loss of thalamic relay neurons or interneuron population in the cortex and hippocampus which are well-defined neuropathologic characteristics of NCL mouse models [171,249]. Initially, two NCL patients were found to carry a single mutation in the *CLCN-6* gene on one chromosome [245]; yet, one of the patients was lately identified to be homozygous for mutation in the *Cln5* gene [252]. CLCN-7 mutations have not been reported yet in NCL disease patients. Nevertheless, it cannot be excluded that mutations in CLCN-6 or CLCN-7 may account for new or rare forms of human NCL disease.

4.3. Development of experimental therapies

NCL mouse models have been used to evaluate the feasibility and efficiency of different therapeutic interventions. An important consideration for the design of a therapy is the solubility of the mutated protein. There are generally two classes of proteins affected in NCL, mutations occur either in a soluble lysosomal enzyme or in a transmembrane protein. The replacement of a soluble enzyme is aided by the phenomenon of cross-correction. Functional enzyme restoration in a small cohort of cells with functional enzyme can be therapeutic as the enzyme can be secreted from expressing cells and taken up by neighbouring cells via the mannose-6-phosphate pathway. The phenomenon does not apply to transmembrane proteins and the currently available techniques

confine functional restoration of these proteins to individual cells reducing the likelihood of a therapeutic effect [253].

A powerful strategy to restore the function of a mutated soluble enzyme is enzyme replacement therapy (ERT) [253,254]. In mice mirroring CLN1 disease intravenous administration of high levels of the Ppt1 enzyme leads to an improved neuropathology in the thalamus, a later onset of motor deterioration and increased survival. Beneficial effects are pronounced in animals treated from birth [255]. Interestingly, major organs take up rapidly intravenously injected Ppt1 enzyme and clearance of autofluorescent material occurs outside the CNS [256]. Thus, this approach may be utilised to treat systemic pathology in NCL mouse models with defective soluble proteins. In *Tpp1*$^{-/-}$ mice, intraventricular and intrathecal deliveries of the Tpp1 enzyme cause an attenuated neuropathology, improved neurological phenotype and longer lifespan [194,257].

In order to achieve a long-term therapeutic expression level of a protein, gene therapy using adeno-associated virus (AAV) appears to be a promising strategy [253]. The viral delivery of *Ctsd* to the brain of mice lacking Ctsd activity prolongs lifespan and diminishes neurological and visceral symptoms [258,259], though ceroid accumulation and microglia activation are not prevented [259]. In *Tpp1*$^{-/-}$ mice AAV-mediated gene therapy leads to increased longevity and better performances in motor or behavioural tasks. Neuropathologically, a reduction in reactive gliosis, autofluorescent inclusions and axonal degeneration is detectable [260–262]. In accordance with ERT on *Ppt1*$^{-/-}$ mice, early AAV treatment appears to be more effective [260]. The first clinical trial on patients suffering from classic late infantile CLN2 disease was carried out and long-term follow-up studies are ongoing [263]. CNS-directed gene therapy in *Ppt1* deficient mice results in reduced autofluorescent material, and improvement in brain histology and behavioural assessment, yet no increase in longevity [264,265]. Surprisingly, intracranial injection of recombinant *Ppt1* combined with bone marrow transplants dramatically enhances motor performance and lifespan, an effect not achieved by bone marrow transplantation alone [266].

Hope for NCL disease therapies has also arisen from work with human stem cells. To prevent xenograft immune rejection, *Ppt1*$^{-/-}$ knockout mice were back-crossed on the NOD (non-obese diabetic)-SCID (severe combined immunodeficiency) background and non-genetically modified human CNS stem cells (huCNS-SCs) were transplanted into the brain at neonatal time points. Following treatment the animals show a reduction in autofluorescent material, neuroprotection of host CA1 neurons and delayed motor coordination loss [267]. A similar study is ongoing for *Tpp1*$^{-/-}$/NOD-SCID mice. Evidence is emerging that grafted huCNS-SCs are able to adopt neuronal fate in immunodeficient *Tpp1*$^{-/-}$ animals. It is under investigation whether the engrafted cells are also capable of delivering TPP1 enzyme to the murine brain [268]. Transplantation of huCNS-SCs has been carried out in infantile and late-infantile NCL patients in the course of a phase I clinical trial. One patient has died without detectable toxic effects towards the transplant; the remaining patients are undergoing post-surgical examination.

The development of therapies is problematic for transmembrane forms of NCL disease as cross-correction does not occur for the majority of transmembrane proteins. ERT, gene therapy and stem cell transplantation have not been described for mutated transmembrane proteins in NCL. However, pharmacological approaches demonstrate therapeutic effects in murine models. In *Cln3*$^{\Delta ex1-6}$ mice low administration of EGIS-8332, an agent that attenuates AMPA receptor activity, or memantine, a NMDA receptor antagonist, leads to better performances in motor tasks [269,270]. Similarly, other pharmacological compounds delay the onset of motor abnormalities in *CLN8*^{mnd} mice; however, beneficial long-term effects are minimal [271–274]. Interestingly, treatment with the immunosuppressant agent mycophenolate motefil significantly reverses motor dysfunction and neuroinflammation in *Cln3*$^{\Delta ex1-6}$ mice [275].

4.4. Summary

Mouse models have enormously contributed to a better understanding of the pathology and progression of the various forms of NCL. Each mouse model displays distinct features mirroring the human disease with behavioural and motor deficits, neuronal cell death and accumulation of storage material. Thorough histologic analyses point to a regional selectivity of pathogenic events in all models. The thalamocortical system seems to be important for the onset of pathological changes since astrocytosis and microglia activation occur first in this region followed by prominent neuronal cell loss. The mechanism underlying neurodegeneration is not understood yet and a range of processes is reported including apoptosis, autophagy and oxidative stress. Another feature present in several models is the molecular changes at the presynapse which may also play a role in neurodegeneration. The detailed description of the disease pathology also provides good parameters for the evaluation of candidate NCL mouse models. To date, no mouse models are available for adult or rare variant forms of human NCL; however several are under investigation.

Considerable progress has been made towards the development of therapeutic treatments for NCL. Now, it is possible to deliver functional protein to the murine brain through ERT, AAV-mediated gene therapy and stem cell transplantation for the major forms of CLN1 and CLN2 disease. Most of these treatments appear safe and cause a delay in the onset and progression of the disease pathology. Interestingly, the combination of gene therapy and BMT leads to sustained motor function improvement and extended lifespan in *Ppt1*$^{-/-}$ mice [266].

Only a few therapeutic studies are available for NCL forms with defective transmembrane proteins. The most promising results are obtained following mycophenolate motefil administration to *Cln3*$^{\Delta ex1-6}$ mice including a reduction in motor deficits and neuroinflammation [275].

It is evident that the current approaches are not sufficient to deliver effectual amounts of functional protein to the CNS in order to prevent disease pathology and premature death of the animals. The main obstacles for therapies targeting the CNS are the large size of the brain and the presence of the blood brain barrier (BBB). Only very small lipid-soluble molecules can passively enter the brain or spinal cord and the vast majority of produced therapeutic substances cannot cross the BBB effectively [276].

One strategy to overcome these difficulties is to enhance AAV-mediated delivery of proteins to the brain. In a mouse model of mucopolysaccharidosis type VII, an inherited lysosomal storage disorder, ventricular injection of recombinant AAV2/4, a virus serotype demonstrated to transduce ependymal cells, resulted in a widespread enzyme secretion and attenuation of CNS deficits [277]. A similar approach reveals promising results in *Tpp1* deficient dogs [89]. Moreover, single intrathecal injections of self complementary (sc) AAV9 vectors indicate an increased efficiency of gene delivery compared to traditional single stranded (ss) AAV vectors. The disadvantage of sc AAV vectors, however, is their limited size for packaging DNA [278].

Intravenously administrated sc AAV9 vectors have recently been shown to be able to cross the BBB with prolonged expression in the CNS, thereby presenting a potential delivery route to the brain for NCL therapies [279,280]. Interestingly, an extensive transduction of the entire CNS was also achieved by intravenous administration of AAV9 in neonatal and foetal mice [281,282]. These findings give particular hope for treatments of early onset and aggressive forms of NCL.

Another interesting insight emerges from a study in *Cln3*$^{\Delta ex1-6}$ mice showing that autoantibodies are capable of overcoming the BBB via a size-selective breach [283]. It is not understood how the integrity of the BBB is compromised; however, it may present a potential delivery route for drug supply to the CNS in NCL. The administration of the immunosuppressant mycophenolate motefil causes a reduction in neuroinflammation and motor deficits in *Cln3*$^{\Delta ex1-6}$ animals [275].

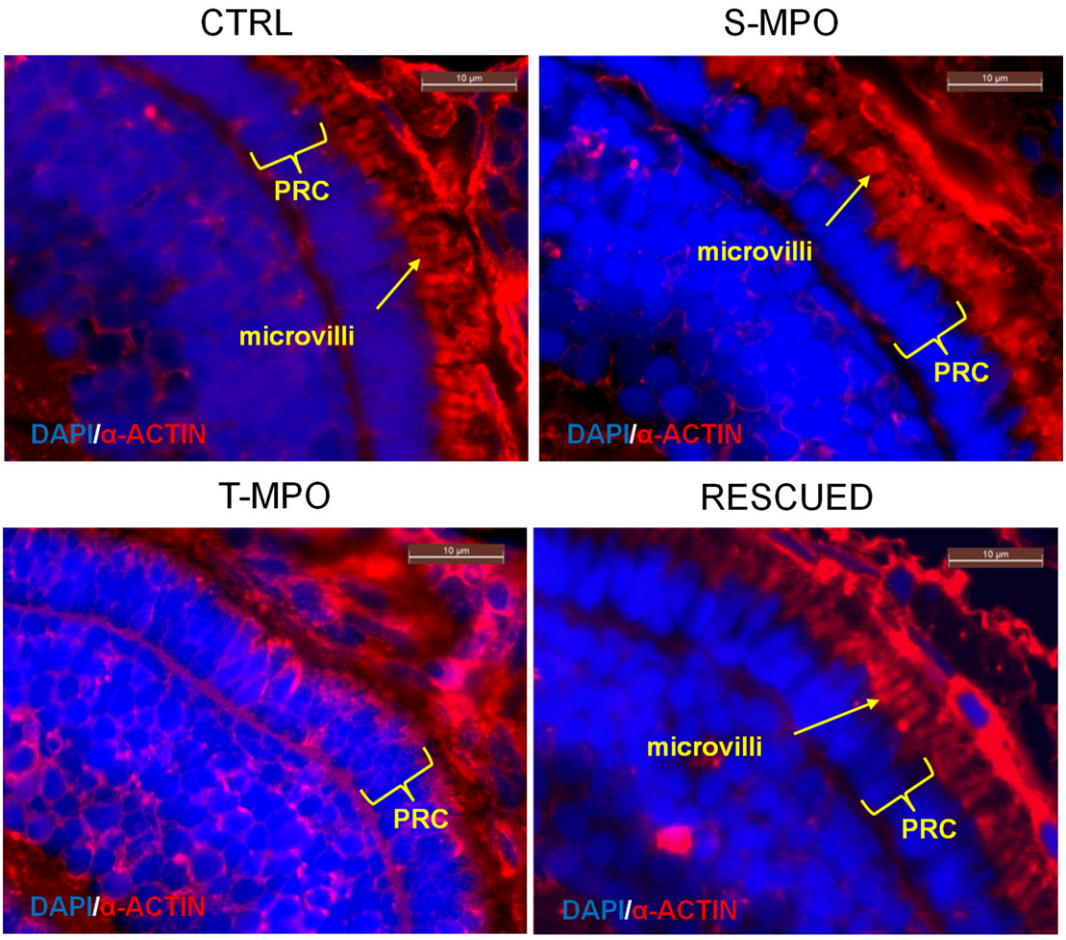

Fig. 2. Immunofluorescence staining of microvilli in the eye of zebrafish following cathepsin D (CD) knock-down and rescue. Immunofluorescence staining of α-actin (red) in eye sections derived from 4 dpf micro-injected larvae (CTRL = control injections; S-MPO = splicing morpholino; T-MPO = translation morpholino; RESCUED = translation morpholino plus 200 pg/egg of mutant CD mRNA). Nuclei are stained with DAPI (blue). The arrow points to the microvilli of retinal pigmented epithelium (RPE) cells. Note the absence of this structure in T-MPO zebrafish. The photoreceptor cell (PRC) layer is indicated by curly brackets. Scale bar is 10 μm. Images representative of five (three for RESCUED) independent experiments.
Reproduced with permission [294].

Currently, a clinical trial is underway to evaluate the safety and tolerance of the agent in juvenile CLN3 disease patients [284].

5. Zebrafish

The zebrafish is a small freshwater tropical fish. The major advantages of this vertebrate is that adults produce large numbers of small offspring, which are externally fertilised, meaning that they can be manipulated and monitored as whole animals from fertilisation onwards. Offspring can be raised to be transparent, enabling an excellent view of internal organs and specific markers, in the live animal. Being diploid, zebrafish have been traditionally used for mutational analysis, enabling researchers to establish the functions of the proteins encoded by each gene that is mutated. In such a way, both forward and reverse mutagenesis screens in zebrafish have proved invaluable for understanding how development is genetically controlled in a vertebrate. However, there are still many hundreds of mutants for which we still do not know the underlying mutation — a potentially rich source of NCL models. Mutation analyses are supported by the sequencing and assembling of the zebrafish genome, although errors persist in the databases (ENSEMBL and NCBI) and require correction by manual annotation. Zebrafish are also amenable to other genetic and chemical manipulations such as knockdown of genes using antisense morpholino oligonucleotides, overexpression of genes using mRNA injection or transgenic constructs, generating mosaics, and modulation of proteins by adding chemicals to their water. Although most of these techniques are possible in adult zebrafish, the embryonic and larval stages are most suitable as they are small, it only takes a few days for animals to reach the right stage for an assay, and they can be cultured and assayed in very large numbers with minimal care in Petri dishes or multi-well plates, meaning that many experiments can be highly-powered. More recently, these techniques have been employed to improve our understanding of the function of disease proteins, to generate disease models, to screen chemical libraries for molecules that modify the phenotype, and to test chemicals for therapeutic efficacy, toxicity and teratogenicity [171]. With the development of automation for the collecting, sorting and arraying of zebrafish embryos and larvae, and of more high-throughput assays, the zebrafish promises to provide an excellent model for drug discovery.

5.1. Zebrafish have homologues of many NCL genes

The first zebrafish NCL gene to be published was *cln2* (now renamed *tpp1*), and since then, only *cln7*, *cln10* and *cln11* have been published. There is one orthologue of each of these genes. Cross-referencing the ENSEMBL database with ZFIN (the zebrafish database) reveals at least one homologue of all known NCL genes (see columns 1–4 in Table 4). There are single gene orthologues of *CLN1*, *CLN2*, *CLN3*, *CLN5*, *CLN8*, *CLN10*, *CLN12*, *CLN13*, *CLCN6* and *SGSH*. However, parts of the zebrafish genome underwent duplication during teleost evolution (reviewed

Table 4
Summary of zebrafish CLN genes and models with confirmed or suggested NCL.

Human gene	Zebrafish homologue (ZFIN)	Zebrafish homologue (ENSEMBL)	Cloning and expression references	Zebrafish model	Genetic mutation	Molecular defect	Phenotype	Disease modeling references
CLN1, Ppt1	ppt1	ENSDARG00000039980	ZFIN	ppt1^{sa9}	Nonsense	Predicted protein truncation after exon2	nd	ZMP
				ppt1^{sa7888}	Nonsense	Predicted protein truncation after exon 3	nd	ZMP
CLN2, TPP1	tpp1	ENSDARG00000042793	ZFIN; [296]	tpp1^{sa11}	Nonsense	Predicted protein truncation after exon 3 (out of 13)	Small eyes and head at 5dpf	ZMP; [293]
				tpp1^{sa2895}	Essential splice site	exon 9 (out of 13)	nd	ZMP
				tpp1^{sa6483}	Nonsense	Predicted protein truncation after exon 10 (out of 13)	nd	ZMP
				tpp1^{hu3587}	Nonsense	Predicted protein truncation after exon 11 (out of 13)	Normal	ZMP
CLN3	cln3	ENSDARG00000037865	ZFIN	cln3^{sa8139}	Nonsense	Predicted protein truncation after exon 12 (out of 15)	nd	ZMP
CLN4; DNAJC5	dnajc5aa	ENSDARG00000042948	ZFIN					
	dnajc5ab	ENSDARG00000004836	ZFIN					
	dnajc5ga	ENSDARG00000041896	ZFIN					
	dnajc5gb	ENSDARG00000017687						
	dnajc5b	ENSDARG00000058147						
CLN5	cln5	ENSDARG00000076339; ENSDARG00000060907 (needs correct annotation)		None				
CLN6	cln6a	ENSDARG00000077584	ZFIN; [123]	cln6^{sa904}	Essential splice site	Exon 6 (out of 7)	nd	ZMP
	cln6b	ENSDARG00000090002						
CLN7, MFSD8	mfsd8	ENSDARG00000015179	ZFIN; [287]	mfsd8^{sa2646}	Essential splice site	Exon 5 (out of 12)	nd	ZMP
				mfsd8^{la018118Tg}	retroviral insertion	nd	nd	[297]
		ENSDARG00000087525		None				
		ENSDARG00000076959		None				
CLN8	cln8	ENSDARG00000075525		None				
CLN10, Cathepsin D	ctsd	ENSDARG00000057698	[298]	CD T-MPO antisense morpholino oligonucleotide	na	Complete protein knockdown	Micropthalmia (RPE microvilli affected), shorter body, swim bladder not inflated, hyper-pigmentation, impaired yolk absorption (all at 4dpf), premature death (10dpf)	[294]
CLN11, Progranulin	grna	ENSDARG00000004954	[288]	grna^{sa6034}	Nonsense	Predicted protein truncation after exon 17, 18 or 22 (out of 19, 24 and 20 exons is each respective transcript)	nd	ZMP
				grna^{sa5206}	Nonsense	Predicted protein truncation after exon 5, 6, or 6 (out of 19, 24 and 20 exons in each respective transcript)	nd	ZMP
				zfPGRN-A ATG MO and zfPGRN-A 5'UTR MO antisense morpholino oligonucleotide	na	severe protein knockdown	Reduced motility, widespread disruption of CNS development, motoneuron axon truncation and premature branching	[289]
				pgrnA ATG MO and pgrnA 5'UTR MO antisense morpholino oligonucleotide	na	nd	motoneuron axon truncation	[290]
	grnb	ENSDARG00000025081	[288]	granulinB GT- grip antisense grip oligonucleotide	na	50% knockdown	No phenotype	[291]
				pgrnB ATG MO and pgrnB 5'UTR MO antisense morpholino oligonucleotide	na			

nd = not reported; na = not applicable.

within [286]) and so there are more than one potential orthologue for the other *CLN* genes — only further analysis of expression and function will allow us to determine which genes are the true orthologues, or if each orthologue has functional subspecialisation. For those with several possible orthologues, the proposed true orthologue is shown in bold in Table 4 (if known). For *CLN7*, Aiello et al. [287] and ZFIN both

consider ENSDARG00000015179 to be the true orthologue, due to the level of sequence conservation. In the case of *CLN11*, *grna*/ENSDARG00000004954 is considered to be the most likely orthologue, based on conservation levels, gene structure, expression and phenotypic analyses [288–290], yet phenotypic analysis suggests that *grnb*/ENSDARG00000025081 might also retain some function of relevance to NCL [290], although an independent knockdown experiment produced no phenotype [291]. For *CLN14*, ZFIN considers *kcnd7*/ENSDARG00000061580 to be the true orthologue due to the level of conservation. A lack of information leaves us unable to determine if any of the *CLN4* and *CLN6* genes are more orthologous than the others.

5.2. Zebrafish models of NCL

The expression of several *cln* genes can be found in publications and on ZFIN (column 4 in Table 4), and the consequences of gene knockdown are also summarised (column 5–9 in Table 4). At the time of writing, very few of the available disease models have been studied (denoted as nd in column 8 of Table 4). Clearly, we still know very little about most of the zebrafish *cln* genes and the consequence of their manipulation, illustrating the lack of exploitation so far of this versatile model organism for NCL research. In the rest of this section, we focus on the studies describing knockdown phenotypes for *cln2* (*tpp1*), *cln10* (*ctsd*) and *cln11* (*grna* and *grnb*).

An image of the zebrafish *tpp1*^{sa11} mutant is reported on the Zebrafish Mutation Project (ZMP) database (http://www.sanger.ac.uk/cgi-bin/Projects/D_rerio/zmp/gene.pl?id=ENSDARG00000042793) and the homozygous mutant has smaller eyes and head compared to its normal siblings at 5 days post-fertilisation (dpf). This phenotype is caused by a premature termination codon (PTC; nonsense mutation) in exon 2. A second mutant, *tpp1*^{hu3587}, however, has no phenotype, presumably because the nonsense mutation is in exon 11 and enough protein of a sufficient length is produced. Abstracts presented at previous conferences [292,293] have demonstrated that the *tpp1*^{sa11} mutant does indeed show the hallmarks of NCL [292,293]. Not only does this provide a validated zebrafish model of CLN2 disease, but we now also know what phenotypes to expect when screening for NCL mutants.

Knockdown of the zebrafish *ctsd* gene was performed by the injection of a *ctsd*-specific antisense morpholino oligonucleotide (CD T-MPO), causing a small eye phenotype, loss of swim bladder, shorter body, hyper-pigmentation, poor yolk absorption and premature death [294]. The authors demonstrated that the small eye was accompanied by the absence of retinal pigmented epithelium (RPE) microvilli at 4 dpf, suggesting that the RPE may not be properly formed or may degenerate in CLN10 disease (Fig. 2). Although the phenotype is consistent with the zebrafish *tpp1* mutant, it is not yet clear whether CD T-MPO-injected fish display the hallmarks of NCL — further investigation will be needed.

Mutations in progranulin were recently shown to cause CLN11 disease, although it was previously demonstrated that autosomal dominant mutations cause frontotemporal lobar degeneration (FTLD). Hence, the few studies on zebrafish Progranulin have focused on its role in FTLD and its association with TAR DNA binding protein-43 [289–291]. However, to explore the function of the zebrafish *progranulin* genes, *grna* and *grnb* were knocked down by antisense oligonucleotide injection, a method which essentially replicates the molecular defect in CLN11 disease. It appears that both *grna* and *grnb* can cause truncation of motoneuron axons [289,290] and *grna* knockdown causes disruption of the CNS early in embryonic development (this was not studied in the *grnb* knockdown). It remains to be seen if motoneuron axon defects exist in CLN11 patients and how well these fish model NCL.

One common theme from these studies is that in zebrafish the *tpp1*, *ctsd10* and *pgrna*/*grnb* genes would be considered to be developmental genes, as phenotypes begin during embryonic stages, suggesting that prenatal changes may occur in humans with these forms of NCL. Alternatively, this may simply reflect the lack of maternal support available to the developing zebrafish.

5.3. Development of experimental therapies

Recently, the *tpp1*^{sa11} mutant was used to determine if premature termination codon readthrough drugs might be a valid therapeutic approach to follow, given that there are two new drugs available that are in clinical trial for cystic fibrosis and Duchene muscular dystrophy. Russell and Mahmood reported that *tpp1*^{sa11} mutants had increased mortality compared to their normal siblings when treated with one of these drugs (Ataluren), with no significant phenotypic improvement [295]. This implies that patients with CLN2 disease may not find this class of drug beneficial and may even find it more toxic than healthy people will. This warrants further examination using mammalian models and patient tissues, and should be kept in mind when prioritising experimental therapies.

5.4. Summary

Although the use of zebrafish for NCL research is still in its infancy, these studies demonstrate the potential of this model organism for disease modelling, mechanistic studies, and experimental therapy testing. However, the specific future contribution of zebrafish studies will largely depend on how well each zebrafish model of NCL replicates the disease and what phenotypes they display at what stage of life. For example, the validated model of CLN2 disease is well-suited to all of those studies as the phenotype presents during embryonic stages and rapidly progresses, while displaying locomotion phenotypes that can be assayed in a high-throughput manner. It is likely that this model, and hopefully other models in the future, will be used in drug discovery for CLN2 disease.

6. Fruitfly, *Drosophila melanogaster*

The fruitfly *D. melanogaster* has a long history of use as a 'gene discovery' organism due to its genetic amenability and short generation time. It has been used extensively in classical genetic studies to identify genes active in a variety of biological processes including development, ageing, cell signalling, cell adhesion, behaviour and learning [299,300]. A large number of the genes identified in these screens have been revealed to have homologues with the same functions in higher organisms including humans, contributing greatly to the identification of the genetic pathways underlying a variety of biology. Recently, *Drosophila* has been increasingly used to both model human disease and elucidate the normal functions of the genes affected in human disease [301]. About 70% of the genes affected in human disease are conserved within *Drosophila* and these genes can be manipulated to generate pathologies similar to that seen in humans [302]. Furthermore, the large number of genetic and cell biological tools available for *Drosophila* allows the manipulation and study of gene function at a fine level in vivo [303]. Many investigations have involved the expression of human disease genes within *Drosophila* to characterise their activity, identify genetic partners in vivo or allow medium throughput screens of modifier compounds [304–306]. In particular, *Drosophila* has been utilised to model a variety of neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, several spinocerebellar ataxias and fragile X syndrome among others [307]. *Drosophila* is now beginning to be used to aid the study of neuronal ceroid lipofuscinosis where it has contributed to the growing understanding of the molecular basis of the disease and may also provide an additional platform for the development of therapies.

6.1. Drosophila contains homologues of a subset of the NCL proteins

Drosophila contains homologues of the genes affected in CLN10 disease (*cathepsin D*; *cathD*), CLN1 disease (*Ppt1*), CLN3 disease (*Cln3*) and CLN7 disease (*Cln7*). It is likely that the *Drosophila* proteins have similar functions to their human counterparts as the *Drosophila* proteins show

strong conservation; *Drosophila* cathepsin D (CathD) has a 65% amino acid similarity [308], Ppt1 has 75% similarity [309], Cln3 shows 60% similarity [310] and Cln7 has 56% amino acid similarity to the human form [311]. The fly Ppt1 also has the same thioesterase activity as the human protein [309]. As with the human proteins it has proven difficult to obtain antibodies to detect the expression patterns of the *Drosophila* NCL proteins in vivo. However, the expression of tagged forms of the proteins reveals that Ppt1, Cln3 and Cln7 are found to be localised to the lysosome, although at high levels of expression some proteins can be detected in additional locations including the plasma membrane [310,312] (Tear Laboratory, unpublished observations). RT-PCR has revealed that *Ppt1* and *cathD* are expressed at low levels fairly ubiquitously [308,309], thus the fly forms display similar properties and expression patterns as the human proteins.

6.2. Drosophila provides models of human NCL disease

Although *Drosophila* does not have homologues of all the genes affected in the different NCLs, it is likely that the NCL genes in *Drosophila* are performing core functions that are necessary for normal neuronal health and their function is likely to be similar to the function of the human genes. *Drosophila* resources are now available for the study of *cathD*, *Ppt1* and *cln3* (Table 5), where the effects of loss or gain of activity of the genes can be studied, while models for *cln7* are being developed. Loss of function mutations in these genes give rise to phenotypes that bear many of the hallmarks of the human disorders. Mutations in *cathD* and *Ppt1* both lead to the accumulation of autofluorescent material within the brain and the deposition of electron dense material. In the CLN10 disease model the storage material is granular with some lamellar elements resembling those seen in ovine congenital NCL [308]. In the CLN2 disease model the deposits are spherical and composed of a granular core surrounded by concentric layers of electron dense material [313]. Similar phenotypes are also observed in mutations in the predicted lysosomal sugar carrier *spinster* (also known as *benchwarmer*) [314–316] although the human homologue of this gene has not yet been associated with an NCL disorder. Thus loss of NCL genes in *Drosophila* results in pathologies related to the human disease. In common with human patients the absence of either the *Ppt1* [313] or *cln3* [317] gene leads to a shortened life span, while loss of *cathD* [308] results in a moderate amount of neurodegeneration and loss of *Ppt1* shows some modest disruption to the CNS [318]. Studies in *Drosophila* have also indicated that the level of expression of the NCL genes is important since targeted overexpression of *Ppt1* or *cln3* in the eye using the UAS-GAL4 system causes neural degeneration resulting in a deformed eye [310,319]. This pathology is dependent on increased NCL gene activity since overexpression of a truncated form of Cln3, similar to that of the common JNCL allele, or mutated versions of Ppt1 lacking enzymatic activity does not show any degeneration. Furthermore, these phenotypes are specific to an increase in NCL gene function since over-expression of other lysosomal proteins, such as Lamp, does not show the phenotype [310]. The overexpression phenotypes are likely to be due to the activation of the same pathways as the human proteins since overexpression of human CLN3 in the *Drosophila* eye also gives rise to the same neurodegenerative pathology. The phenotypes induced by Cln3 overexpression, in the wing as well as in the eye, are indicative of an impact on Notch and JNK signalling [310]. Further large scale genetic screens have begun to reveal a common role for the *Drosophila* NCL genes in signalling and membrane trafficking and indicate that they are required for normal synapse function (see below). Similar roles have been previously suggested for *spinster*, as mutations in *spinster* lead to defects in endocytosis and synapse function [314–316]. These observations overlap with those emerging from a number of vertebrate studies that suggest that synaptic failure may be a common early pathology in NCLs [175,185–187].

6.3. Genetic screens in Drosophila identify pathways requiring NCL gene function

Since the identification that the *Drosophila* NCL genes show very similar properties to the human forms recent work has focused on the identification of genetic partners and pathways that require NCL gene function. This has been performed by second site genetic modifier screens, which allow the unbiased identification of genes that modify the phenotypes caused by overexpression or loss of function of the *Drosophila* NCL genes when they are also knocked out or overexpressed. Large scale screens have been performed to identify modulators of Ppt1 and Cln3 activity [317,320,321], whereas a smaller scale investigation has been performed to identify candidate partners for cathepsin D [322]. To simplify mass screening the large scale screens have sought genes that modify the degeneration of the eye caused by overexpression of Ppt1 or Cln3. These screens have identified twenty-seven genes that modulate the activity of Ppt1 and thirty-eight genes that modulate the activity of Cln3. Although the same genes have not been identified in the different screens, it is clear that modulation of several common biological pathways influences the function of Ppt1 or Cln3. The major activities identified in the screens include genes required for endocytosis and membrane trafficking (e.g. *endophilin A*, *srp9*, *blue cheese*), genes required for protein stability (e.g. *fat facets* (a ubiquitin protease), *CG5823* (an E2 ubiquitin conjugating enzyme)), genes required for signalling and transcription (e.g. *saxophone* (TGF-B receptor type I), *kayak* (fosB)), RNA stability or processing (e.g. *mago nashi*, *boule*) and genes required for stress response (e.g. *Hsc70-3*, *mekk*, *JafRac*). Since many of the genes fall into a relatively small number of classes it appears that the NCL genes may function within similar cellular processes. The failure of the screens to identify the same genes could be due to the screening of different mutant collections or that the different NCL genes affect similar biology via different pathways.

Not only do these genes modify phenotypes generated by an increase in NCL gene function but, where they have been tested, the same genetic pathways also appear to be affected in the *Drosophila* models mimicking the recessive disorders. Endocytosis is significantly compromised in *Drosophila* lacking *Ppt1* function, where uptake of fluorescent avidin or HRP is impaired, the tracer is not efficiently transferred to the lysosome and the ultrastructure of specialised membranes necessary for endocytosis is abnormal [320]. Additionally a *Drosophila* model of CLN3 disease, which lacks Cln3 activity, is hypersensitive to oxidative stress. These animals have a reduced ability to survive exposure to reactive oxygen species (ROS) and show a slight increase in ROS levels under normal conditions. It appears that the *cln3* mutant animals remain able to sense an accumulation of ROS but are unable to mount the appropriate response [317].

A smaller scale screen looking for genetic partners for *cathD* made use of a model of CLN10 disease. Loss of *CathD* function in *Drosophila* causes a mild degeneration of the retina and this pathology could be enhanced by reducing the activity of *shibire*, *heat-shock cognate 4*, or *thioredoxin reductase-1* or by increasing the activity of *target of rapamycin* or *Columbus*, an HMG-Co-reductase, suggesting that loss of *cathD* may also affect endocytosis or reduce the animal's ability to combat oxidative stress leading to potential failure in lipid metabolism [322]. Overexpression of *shibire*, the *Drosophila* dynamin, also enhances the Ppt1 overexpression phenotype revealing a potential common pathway requiring Ppt1 and cathepsin D [320].

6.4. Drosophila models indicate a role for CLN genes at the synapse

The *Drosophila* models have identified a discrete number of cellular processes where changes may lead to the symptoms associated with the NCLs and candidate genes that could mediate the disease. Although many of the processes have been identified using non-neuronal tissues these *Drosophila* studies implicate NCL gene function with roles in

Table 5
Summary of *Drosophila* NCL models and transgenics for studying NCL.

Human gene	*Drosophila* strain	Genetic mutation	Molecular defect	Phenotype	Reference
Cathepsin D, CLN10	*CathD*^{1}	Deletion of exon1	70% of protein coding region deleted	Storage material, autofluorescent inclusions, mild neurodegeneration	[308]
	UAS:CathD	None	Overexpression construct	None reported	[208,322]
Ppt1, CLN1	*Df(1)446-20*	Chromosomal deletion of *Ppt1* and 3 additional genes	No enzyme activity	Storage material, autofluorescent inclusions, shortened lifespan	[309,313]
	Ppt1^{S77F}	Ser 77 to Phe missense	No enzyme activity	Storage material, autofluorescent inclusions, shortened lifespan	[313]
	Ppt1^{A179T}	Ala 179 to Thr missense	No enzyme activity	Storage material, autofluorescent inclusions, shortened lifespan	[313]
	UAS:Ppt1-RNAi$^{8/7}$	RNA interference	10% enzyme activity	Some storage material	[313]
	UAS:Ppt1-8.1	None	Overexpression construct	Photoreceptor degeneration, apoptosis	[319]
	UAS:Ppt1-S123A	Ser 123 to Ala missense	Catalytic inactive overexpression construct	None	[319]
CLN3	*Cln3*$^{\Delta MB1}$	Deletion of exon 1 & 2	N terminal deletion	Reduced life span, hypersensitive to ROS	[317]
	UAS:Cln3	None	Overexpression construct		[310]

endolysosomal trafficking, vesicle cycling, BMP and JNK signalling, RNA metabolism and oxidative stress. Several of these roles are also emerging from vertebrate NCL models and defects in these processes are linked to other neurodegenerative disorders [185,323]. Many of these roles are required at the synapse where they act as important regulators of synaptic function. Synapse vesicle and membrane trafficking are essential for normal synapse function both for signal transmission and synapse plasticity. The latter is monitored via BMP signalling between the pre and post synaptic partners [324] and can be modulated by controlling gene expression from ribonucleoprotein particles sequestered at the synapse [325,326]. The synapse is also sensitive to oxidative stress and the impairment of ROS clearance can affect synapse function [327,328]. Thus studies using *Drosophila* as a model for NCL have led to the emerging hypothesis that the NCLs may be a consequence of synaptic deficits leading to neuronal dysfunction. Recent work has begun to reveal that *cln3* and *cln7* may both be required to maintain synaptic morphology, as the larval neuromuscular junction synapse fails to mature appropriately in the absence of either of these genes (Tear laboratory, unpublished observations). This hypothesis and the roles of the genes identified as mediators of the disease need to be verified and tested in vertebrate models yet it is likely that the *Drosophila* models will have revealed key genetic pathways affected in the disease and opened up potential new therapeutic targets. In the future *Drosophila* will continue to give insight into the pathways where the NCL genes function and also provide a novel platform for the testing and development of therapies.

7. Yeast

The budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* are two of the best-characterised eukaryotic model systems. Both are highly amenable to genetic manipulation and analysis, have short generation times and reproduce in a genetically stable manner. In addition, yeast recapitulates many fundamental aspects of mammalian cell biology. These experimental advantages have motivated the development of an array of tools for the analysis of gene function and cell biology in these yeast species, including genome wide knock-out and GFP-fusion libraries. Consequently, yeast has been extensively utilised as an experimental system, and is rapidly emerging as a powerful model for the cell biology of neurodegeneration [329].

7.1. Yeast models for NCL

S. cerevisiae and *Sz. pombe* have both been proven as valuable tools in the investigation of NCL biology. *S. cerevisiae* contains orthologues of the NCL genes *CLN3* and *CLN10*, whereas *CLN1*, *CLN3* and *CLN10* are conserved in *Sz. pombe* (Table 6). The budding and fission yeast orthologues of *CLN3* (*BTN1* and *btn1* respectively) have been studied extensively. In work prior to 2006, *S. cerevisiae BTN1* had been implicated in the regulation of vacuolar (the yeast lysosome) and cellular pH, basic amino acid homeostasis and nitric oxide production [330–336]. *Sz. pombe btn1* has been reported to influence vacuolar pH and morphology, cell polarity, cell wall deposition, heat tolerance and osmoregulation [337–339]. *PEP4*, the budding yeast orthologue of *CLN10*, is also well-characterised, and known to mediate vacuolar protease maturation and vacuolar morphology (reviewed in [340]). The fission yeast *CLN1* (*ppt1*) and *CLN10* (*sxa1*) orthologues have received little attention since their initial identification and they are featured in only a small number of early studies [341–343].

7.2. Modelling CLN3 disease in yeast

The now well-established yeast phenotypes associated with loss of *btn1* function have been exploited to probe the function of *btn1* carrying disease-causing mutations. In a study by Haines et al. [344], fission yeast expressing a range of such mutants was examined for the rescue of a bank of marker phenotypes. Using this approach it was indicated that a mutation, equivalent to the common intragenic 1 kb deletion in humans, retains some function and is able to rescue defects in vacuolar size. Mutations in the luminal face of the protein, in particular the amphipathic helix of the third luminal domain, had the most severe effect upon function. Finally, the Btn1p^{E240K} mutant, which models a mutation (Glu295Lys) associated with a protracted disease progression, was the only mutant able to rescue cell curving, potentially indicating a significant retention of function.

In another study that expanded upon known phenotypes of cells lacking *BTN1*, Wolfe et al. [345] further probed the link between *BTN1* and cellular pH in *S. cerevisiae*. The study indicated that BTN1p underwent pH-dependent changes in localisation, glycosylation and expression levels. Expression of *BTN1* was higher at pH 6.0 compared to pH 4.0. The increase in BTN1p at pH 6.0 consisted exclusively of a glycosylated form of the protein, and it was suggested that this glycosylation was key to protein stability under these conditions. The change in post-translational modification was also accompanied by an alteration in protein localisation. At pH 6.0, BTN1p co-localised with the vacuolar V-ATPase at the vacuolar membrane. Yet, upon growth at pH 4.0, BTN1p redistributed to unidentified cytosolic puncta. These findings highlight the interplay between BTN1p, a protein known to be involved in pH regulation, and environmental pH.

Further work has also explored this relationship [346]. Using yeast two-hybrid protein interaction, co-immunoprecipitation and co-localisation, BTN1p was shown to interact with SDO1p, an orthologue of the protein mutated in Shwachman–Bodian–Diamond syndrome.

Loss of SDO1p resulted in decreased vacuolar pH and decreased vacuolar V-ATPase activity. These phenotypes were exacerbated by *BTN1* overexpression or inhibition of proton transport, and could be recapitulated under both these conditions in the presence of SDO1p. Based upon these observations, it was suggested that BTN1p regulated V-ATPase activity, negatively influencing the coupling of ATP hydrolysis to proton pumping. More recent work, however, indicated that there was no direct interaction between BTN1p and the V-ATPase [347]. Finally, due to the role of SDO1p in ribosomal maturation, it was suggested that this pathway was key to vacuolar function [346].

The experimental advantages inherent in the yeast model system lend it to the analysis of global regulatory change, and the *Sz. pombe* model for CLN3 disease has been used in this way to investigate the metabolome of cells lacking *btn1* [348]. Positive and negative changes were observed in amino acid levels, in addition to a decrease in certain nucleotides upon loss of *btn1* function. The concentration of glucose in spent growth media from cells lacking *btn1* was also decreased compared to wild-type control strains, suggesting an increased glycolytic flux, and markers for TCA cycle activity were increased. Supplementation with glycolytic substrates rescued the temperature-sensitive growth defect of cells lacking *btn1*, suggesting that increased glycolysis may be an adaptive change. Detailed analysis of these global metabolic changes may provide new insights into the role of *btn1* and, as a consequence, *CLN3*.

The emerging role of *CLN3* and its orthologues in membrane and protein trafficking has received significant attention in recent years. The link was first explored in mammalian systems, where it was demonstrated that overexpression of *CLN3* caused aggregation of Hook1, a protein known to regulate endocytosis [349]. The *S. cerevisiae* orthologue of *Hook1*, designated *BTN2* due to its up-regulation in the absence of *BTN1* [350], had already been implicated in vesicular targeting in budding yeast [351], and was subsequently retrieved as an interacting partner of the endocytic v-SNARE SNC1p [352]. The same study demonstrated that BTN2p influences transport from the Golgi through the endosome, and probably export from the late endosome. It was also suggested that BTN2p may be necessary for the retrograde transport of certain cargoes back to the Golgi.

A study in *Sz. pombe* was the first to explore such processes in a yeast model for CLN3 disease [353]. In this yeast the loss of Btn1p was shown to cause missorting and secretion of the vacuolar hydrolase carboxypeptidase Y (Cpy1p), a result of the retention of the Cpy1p receptor Vps10p in the Golgi. Analysis of a number of double deletion strains, defective for vacuolar protein-sorting, demonstrated that Btn1p was also involved in Vps10p-independent Cpy1p-sorting pathways.

Such observations would suggest that Btn1p has a direct influence upon the secretory pathway. Indeed, the same study demonstrated that Btn1p was located at the Golgi apparatus, and directly influenced Golgi number, subcellular localisation and morphology [353]. This influence was later explored in the budding yeast model for CLN3 disease [354]. Loss of *BTN1* resulted in a similar phenotype to loss of *BTN2*, preventing the retrieval of certain cargoes from the late endosome to the Golgi. In addition, it was demonstrated that BTN1p localised to the Golgi, which required transport from the late endosome. BTN1p also directly influenced the assembly of Golgi SNARE complexes, negatively regulating the SNARE SED5p. This influence appeared to be due to the phosphorylation of SED5p. In addition, BTN1p influenced Golgi morphology, with the loss of BTN1p, and SED5p phosphorylation, causing increased Golgi clustering. Finally, BTN1p was shown to influence SED5p phosphorylation through the palmitoylated kinase YCK3p, possibly by modulating its membrane anchoring.

Correct Golgi function is also vital to the metabolism and distribution of membrane lipids. A recent study has explored the potential role of *BTN1* in phospholipid distribution in *S. cerevisiae* [347]. Loss of *BTN1* led to a decrease in the levels of phosphatidylethanolamine (PtdEtn) in mitochondrial and vacuolar membranes. Deletion of *BTN1* and *PSD1*, the mitochondrial phosphatidylserine decarboxylase primarily responsible for PtdEtn synthesis, caused a further decrease in PtdEtn levels, suggesting that these two proteins may be acting at parallel pathways. A similar but less pronounced effect was also seen with the Golgi/endosome associated phosphatidylserine decarboxylase *PSD2*. A decreased rate of PtdEtn transport from the ER to the mitochondria and vacuoles was also observed in these double deletion strains. Ethanolamine supplementation did not increase PtdEtn concentration and, at higher concentrations, was toxic to cells lacking *BTN1*, suggesting impairment of ethanolamine incorporation through the Kennedy pathway. Finally, it was suggested that these alterations in phospholipid composition could account for the impaired function of certain membrane bound proteins in cells lacking *BTN1*, such as the vacuolar V-ATPase.

The regulatory link between *BTN1* and *BTN2* helped to identify membrane and protein trafficking as a potential function for *BTN1*. This link could also prove fruitful in future analysis of *BTN1* activity. A number of recent studies have linked *BTN2*, and its newly identified negative regulator *BTN3*, to the cellular stress response and prion

Table 6
Summary of yeast NCL models.

Human gene	Yeast species	Yeast strain	Genetic mutation	Molecular defect	Phenotype	Reference
Cathepsin D, CLN10	*S. cerevisiae*	*PEP4Δ*	*PEP4Δ::HIS3*$^{+}$ *PEP4Δ::kanMX4* (Euroscarf deletion library)	Complete deletion	Altered vacuolar morphology, decreased vacuolar protease maturation, protein turnover and post-mitotic life span (increased apoptotic and necrotic cell death)	[340,357]
	Sz. pombe	*sxa1Δ*	*sxa1Δ::ura4*$^{+}$	Complete deletion	Increased sensitivity to mating pheromone	[341,342]
Ppt1, CLN1	*Sz. pombe*	*pdf1*^{S106A} *pdf1*^{D226A}	*pdf1Δ::his3*$^{+}$ *pAAL-pdf1*^{S106A} *pdf1Δ::his3*$^{+}$ *pAAL-pdf1*^{D226A}	Complete deletion of the *pdf1* locus, consisting of *ppt1* and the essential domain *dolpp1*. The expression plasmids *pAAL-pdf1*^{S106A} and *pAAL-pdf1*^{D226A} restore *dolpp1* function but lack *ppt1* function.	Increased sensitivity to vanidate and alkaline pH	[343]
CLN3	*S. cerevisiae*	*BTN1Δ*	*BTN1Δ::HIS3*	Complete deletion	Reduced nitric oxide production, alteration in vacuolar and cellular pH, basic amino acid homeostasis, trafficking and phospholipid distribution	[330–336,345–347,354]
	Sz. pombe	*btn1Δ*	*btn1Δ::leu2* *btn1Δ::ura4*	Complete deletion	Alterations in vacuolar pH and morphology, cell polarity, cell wall deposition, heat tolerance, osmoregulation, trafficking, Golgi size and number, glycolytic flux, amino acid and nucleotide homeostasis	[337–339,348,353]

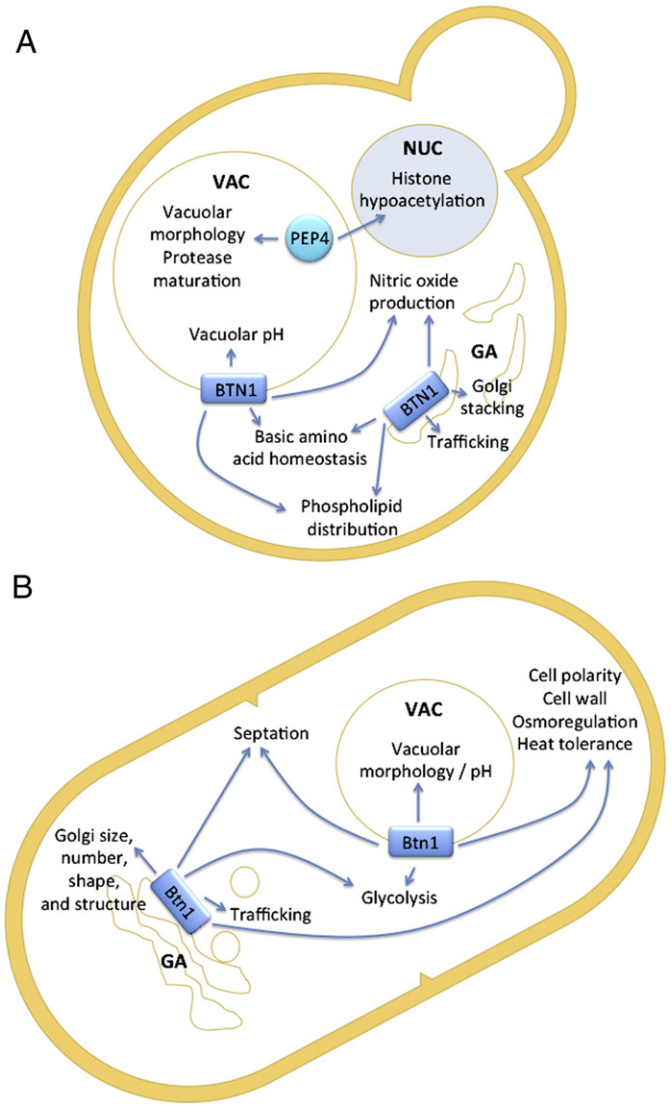

Fig. 3. Established roles for NCL gene orthologues in yeast. Budding yeast (A) BTN1 and fission yeast (B) Btn1 are known to localise to the vacuolar (VAC) and Golgi (GA) membranes. Likely from the vacuolar membrane, BTN1 is known to influence vacuolar pH and Btn1 both vacuolar pH and morphology. The Golgi is the probable site from which BTN1 affects membrane and protein trafficking and Btn1 trafficking in addition to Golgi size and number. In budding yeast, BTN1 also regulates nitric oxide production, basic amino acid homeostasis and phospholipid distribution, although the site at which these occur is less apparent. Similarly, Btn1 also regulates septation, glycolysis, cell polarity, cell wall biogenesis, osmoregulation and heat tolerance. Budding yeast PEP4 is a soluble vacuolar hydrolase, which influences vacuolar morphology and directly regulates protease maturation. The PEP4 propeptide also has an anti-necrotic effect, causing histone hypoacetylation in the nucleus (NUC).

curing [355,356]. Such activities could be of great relevance to neurodegeneration, and may prove important in *BTN1* function.

7.3. Modelling CLN10 disease in yeast

Neurodegeneration is often viewed as a shortening of the chronological, or post-mitotic, lifespan of a cell. Yeast is an ideal system for the study of chronological ageing, as quiescence can be easily induced by carbon or nitrogen limitation. This system has yielded some important insights into the role of *PEP4*, the *S. cerevisiae* orthologue of *CLN10*. *PEP4* is key to maintaining viability in quiescent culture [357]. In addition, *PEP4* expression increased with chronological age, and a loss of *PEP4* was associated with an increase in the oxidative damage of cellular protein. The role of *PEP4* in the turnover of oxidised protein was also highlighted by its up-regulation in response to exogenous oxidative insults.

The positive influence of *PEP4* upon lifespan can be divided into two processes. As discussed, the proteolytic turnover of damaged protein by PEP4p promotes cell survival, delaying apoptotic cell death. However, catalytically inactive PEP4p also improves survival in quiescent yeast populations by delaying necrotic cell death [358]. This activity was conferred by the inactive PEP4p pro-peptide, and correlated with histone hypoacetylation, and the nuclear retention of pro-necrotic factors. These observations could prove highly relevant to NCL, as oxidative stress-induced cell death is often associated with neuronal loss.

7.4. Summary

The conservation of these NCL genes from yeast to humans suggests a fundamental role in eukaryotic cell biology. The use of simple unicellular systems such as yeast therefore represents a powerful tool to investigate the basic cellular changes that occur upon loss of gene function. This approach has already helped to assert a role for *CLN3* in lysosomal homeostasis, protein trafficking, phospholipid distribution and the regulation of metabolic processes, and has highlighted the importance of *CLN10* in post-mitotic survival (summarised in Fig. 3). Given that much is still not known about the basic function of these genes, or the consequences of their loss, yeast will continue to provide a valuable model for the basic cell biology of NCL.

8. Concluding remarks

The range of model organisms used for NCL research is clearly providing a wealth of information about protein function/dysfunction and pathomechanisms, as well as providing new candidate NCL genes and in vivo methods for testing a range of experimental therapies. The studies presented highlight the advantages of each organism and how each of these attributes can be exploited further in future research, providing a powerful arsenal of complementary tools to aid the search for and testing of NCL therapies.

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