Selectivity and Types of Cell Death in the Neuronal Ceroid Lipofuscinoses (NCLs)

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Cloning of the individual genes that are mutated in the neuronal ceroid lipofuscinoses (NCLs), or Batten disease, has opened up new avenues of research into the pathogenesis of these fatal autosomal recessive storage disorders. Genetically accurate mouse models have now been generated for each major form of the disorder, together with several variant forms. Ongoing analysis of these mice is revealing significant new data about the staging and progression of disease phenotypes. Combined with data from human autopsy tissues and large animal models, it is now clear that neurodegeneration is initially selective in the NCL CNS, targeting specific regions and particular cell populations. There is also evidence of selective glial activation that appears to precede obvious neurodegeneration, becoming more widespread with disease progression. Currently, there is debate over the mechanisms of cell death that operate in each form of NCL, with evidence of both apoptosis and autophagy. It is likely that these mechanisms may encompass a spectrum of cell death events, depending upon the specific context of each neuronal population. Taken together, these data have significant clinical implications for the development and targeting of appropriate therapeutic strategies, and for providing the landmarks to judge their efficacy.

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INTRODUCTION

The neuronal ceroid lipofuscinoses (NCLs) are a significant cause of neurological deterioration during childhood (18). Collectively, this group of up to eight genetically distinct lysosomal storage disorders (CLN1-CLN8) is considered the most common pediatric neurodegenerative disease (15, 25, 38). These disorders are typified by their progressive nature, presenting with visual disturbances leading to blindness, neurocognitive and physical decline, an increased severity of untreatable seizures and ultimately premature death (23, 26, 38, 71, 84, 108). Cases usually present during childhood with an infantile (INCL), late infantile (LINCL) or juvenile (JNCL) onset; although rare adult onset forms (ANCL) and an increasing number of variant forms are also recognized (15, 38). The hallmark of NCL pathology is the aberrant progressive accumulation of proteinaceous storage material in lysosomes, with the composition and ultrastructural appearance of this material apparently correlating with disease subtype (38). In late-infantile (CLN2, CLN5, CLN6, CLN8) and juvenile forms (CLN3) the major component is subunit c of the mitochondrial ATP synthase complex (F0F1-ATPase) (74). Infantile NCL (CLN1) has a unique composition predominated by sphingolipid activator proteins A and D (95). This accumulation of storage material occurs in many cell types, but cell death appears specific to central nervous system (CNS) neurons and neural retina. Indeed, there is no evidence for direct link between storage material accumulation and subsequent cell death.

The identification of 6 individual "CLN" genes mutated in different forms of NCL (22, 42, 82, 86, 88, 101, 106) has accelerated research into the underlying pathological mechanisms. Current information on the functions of the NCL gene products is reviewed elsewhere in this issue. Cloning of these genes has also enabled the construction of a variety of mouse models that recapitulate each of the major forms of human NCL (16, 30, 48, 54, 70). Detailed characterization of these models, together with newly validated naturally occurring mutant mouse (13, 22, 56, 82, 106) and large animal models (73) is now underway. Despite this activity, the precise events that lie downstream of these mutations and how these result in the devastating clinical presentation of these disorders remain unclear.

The great advantage of animal models of NCL is the ability to study the progressive development of neuropathology during the course of disease, data which cannot easily be obtained in affected individuals. The emergence of genetically accurate models of the disease has provided a wealth of novel data that describe selective events that range from regional, through to cellular and subcellular levels (15). Comparison of findings from these models to human autopsy material is starting to reveal significant information about the selectivity of pathological events in the NCL CNS (15). Nevertheless, major questions about pathogenesis remain unanswered, including the precise nature of this selectivity and the mechanisms that trigger cell death.

This review focuses upon neuropathological findings from the recent characterisation of animal models, together with emerging data of functional roles for the CLN proteins within the CNS. Successfully combining this knowledge will be essential for devising new and novel palliative or therapeutic treatments. Furthermore, a greater understanding of the type and selectivity of cell death mechanisms that operate in the NCLs will form the basis of landmarks by which the therapeutic efficacy of these approaches will ultimately be judged.

Mouse model		CNS Neuropathology			Retina	References
Gene and protein product	**Design, strain and mutation**	**EM ultratsructure, neuronal degeneration and loss**	**Reactive gliosis**	**Brain atrophy**	**Retinal defects**	
Infantile NCL mouse models						
CLN1/PPT1; Palmitoyl protein thioesterase 1	Knock-out, Mixed C57BL/6J-129/Sv. Neo gene plus in-frame stop codon in exon 9	Classic INCL granular osmiophilic deposits. Ballooned hippocampal and cortical neurons (TUNEL positive), Purkinje cell loss. Selective interneuron loss	Widespread astrocytosis, localized microglial activation	Cortical/subcortical atrophy. Selective cortical thinning and laminar effects	ERG diminished; possibly compromised by poor vision strain background	(30) and unpublished data
CLN1/PPT1; Palmitoyl protein thioesterase 1	Knock-out	Classic INCL granular osmiophilic deposits. Loss of interneuron populations		Marked cortical atrophy	Early visual deficits and blindness by 3-4 mo.	(54)
Late-infantile NCL mouse models						
CLN2/TPP1; Tripeptidyl peptidase I	Knock-in					Dr D. Sleat, pers. comm. Also see (49)
CLN5; CLN5	Knock-out	LINCL type fingerprint and curvilinear profiles		Significant cerebellar atrophy	Visual deficits from 3-4 mo.	(54)
CLN8 (*mnd*); CLN8	Spontaneous mutant C57BL/6.KB2/Rn substrain. c.267-268insC. Frameshift after Val89 (82)	Pentalaminar membraneous profiles. Progressive loss of hippocampal and cortical interneuron populations.	Marked GFAP staining at 9 mo.	Atrophy of the cortex, but not cerebellum at 9 mo.	Retinal storage and degeneration as early as p15, photoreceptors nearly all lost by 8 mo. ERG extinguished by 6 mo. Blind by 2-5 mo.	(3, 4, 7, 8, 9, 13, 67, 76)
CLN6 (*nclf*); CLN6	Spontaneous mutant, Mixed C57BL/6J/10J, C3HeB/FeJLe. c.307insC frameshift after Pro120 (22, 106)	Pentalaminar membraneous profiles. No necrotic or apoptotic neurons seen. Progressive loss of hippocampal and cortical interneuron populations.	Marked GFAP staining at 6 mo.	Cortical atrophy	Storage accumulation in retina by 3 mo. Retinal degeneration similar to mnd but slower by ~2 mo.	(8)
Juvenile NCL mouse models						
CLN3; CLN3	Knock-out, 129/Sv inbred. Neo gene replaces exon 1-6	Classic JNCL multilamellar membraneous fingerprint profiles. Progressive loss of hippocampal and cortex interneuron populations.	Astrocyte/microglia markers upregulated pre-symptoms	Delayed cortical atrophy and thinning	Progressive storage from 1 mo. Mild photoreceptor apoptosis but no extensive degeneration up 20 mo. ERG normal up to 11 mo. Optic nerve degeneration (85)	(70, 87) and unpublished data.
CLN3; CLN3	Knock-out, Mixed C57BL/6J-129/Sv. Neo gene replaces exon 7-8.	11 wks stacked membrane whorls in cortical neurons				(48)
CLN3; CLN3	Knock-in, Mixed 129/Sv-CD1. Deletion of exon 7-8, loxP site left in intron 6.	Classic JNCL fingerprint profiles. No obvious cell changes reported. TUNEL staining negative at 10 mo.	Marked GFAP staining in brain at 10 mo.	No obvious changes in brain architecture up to 17 mo.	Fluorescent retinal deposits from P1.5. Retinal degeneration with photoreceptor loss at 10-17 mo. (possibly linked to strain background)	(16)

Table 1.

Mouse model	Neurological behavioural disorder			References
Gene and protein product	General	Motor	Seizures	
Infantile NCL mouse models				
CLN1/PPT1; Palmitoyl protein thioesterase 1	Viable, fertile. Lack of grooming from 4-5 mo. Aggression and dermatitis. Survival 50% by 7 mo., all dead by 10 mo.	Progressive gait abnormality from 5 mo. Progressing to hind-limb paralysis. Clasping phenotype from 21 wks, 100% by 8 mo.	Frequent myoclonic seizures from 3-4 mo. Some tonic-clonic convulsions	(30) and unpublished data
CLN1/PPT1; Palmitoyl protein thioesterase 1	Viable, fertile. Dead by 6-9 mo.	Progressive motor abnormalities		(54)
Late-infantile NCL mouse models				
CLN2/TPP1; Tripeptidyl peptidase I	Early death	Ataxia		Dr D. Sleat, pers. comm. Also see (49)
CLN5; CLN5	Viable, fertile		Seizures from 3-4 mo.	(54)
CLN8 (*mnd*); CLN8	Viable, fertile. Increased activity and aggression. Learning and memory deficits. Dermatitis. Dead by 10-12 mo.	Progressive motor deterioration from 4-5 mo. leading to ataxia, paralysis. Immobile by 9-12 mo. Rotarod and various other deficits, abnormal clasping from 5 mo.	None apparent	(3, 4, 7, 8, 9, 13, 67, 76)
CLN6 (*nclf*); CLN6	Viable, fertile. Clinically normal until 8 mo. Dead by 12 mo.	From 8 mo. develop spastic rear limb paresis progressing to paralysis, typical of upper motor neuron degeneration. Abnormal clasping	Death of a few mice by terminal seizures reported	(8)
Juvenile NCL mouse models				
CLN3; CLN3	Viable, fertile. Autoimmune response (10). Dermatitis from 16 mo. with irritation and scratching. Dead by 18-20 mo.	Motor deterioration seen by 16 mo. with reduced activity, stiff and slow gait		(70, 86) and unpublished data.
CLN3; CLN3	Viable, fertile. Lower mating success in knockouts. Some mutants show circling behaviour from 8 wks	By 25 wks stiff and slow gait	No seizures up to 25 wks	(48)
CLN3; CLN3	Viable, fertile. Reduced lifespan from 7 mo., 80% survival at 10-12 mo.	Abnormal gait at 10-12 mo. 42% show clasping phenotype at 10-12 mo.		(16)

Table 2.

ANIMAL MODELS OF NCL

Mouse models of NCL. Mice can be genetically modified with relative ease and have accordingly become the primary model organism for investigating NCL pathogenesis. At present, 7 different NCL mouse strains have been developed which carry a "knock-out" mutation to abolish gene function or a "knock-in" recreation of a human disease-specific mutation (Table 1). Together with 2 spontaneous mutants carrying defined NCL gene mutations, these mice represent models for each of the 6 genetically identified forms of NCL. All 9 mutant strains have progressive neuronal storage disorders that resemble NCL with widespread intracellular accumulation of autofluorescent material in neurons and other cell types (Figure 1), and different degrees of neurodegeneration (Table 1). The clinical course and age of death in each model broadly reflect the corresponding human NCL subtype (Table 2).

Two different PPT1 knock-out mouse models of infantile NCL have been produced using different targeting strategies (30, 54). These models exhibit an aggressive NCL phenotype with severe cortical atrophy, retinal degeneration, spontaneous seizures and early death by 6 to 10 months of age (30, 54). More than one group have tried to generate a TPP1 mouse model for classic LINCL (49), but this has proved technically difficult and due to the local genomic structure has required a more complicated gene targeting strategy. However, this approach has recently been successful and TPP1 deficient mice appear to be severely affected with a course characterised by motor deficits and early death (Sleat, personal communication).

Mouse models now exist for the variant late-infantile NCL forms CLN5, CLN6 and CLN8. Preliminary data from CLN5 null mutant mice reveal significant brain atrophy, seizures and visual deficits (54), although these mice await detailed characterization. There are 2 spontaneously occurring mouse mutants, nclf and mnd, which carry severe frameshift truncation mutations predicted to severely affect or abolish gene function, in the genes orthologous to CLN6 and CLN8 respectively (22, 82, 106). The mnd mouse is well characterized with a phenotype of brain atrophy, neurological and motor deficits, retinal degeneration, and premature death at 10 to 12 months of age (3, 7, 13, 67, 76). Data are more limited for the nclf mouse which has a similar but more protracted clinical course compared to the mnd mouse, exhibiting comparable symptoms with a delay of about 2 months (8, 56).

Three mouse models now exist for CLN3, with 2 different targeting strategies used to generate CLN3 null mutant mice (48, 70), and a knock-in approach

used to recreate the major 1 kb deletion present in the majority of JNCL patients (16). Although this deletion is predicted to be a severe frameshift truncation mutation, immunohistochemical staining in these CLN3$^{\Delta ex7/8}$ mice suggests that a truncated mutant protein is stably expressed, albeit at reduced levels. All three CLN3 mutants display a later-onset neurodegenerative phenotype than the other NCL models with retinal and motor deficits, consistent with juvenile NCL (16, 29, 48, 70). Neurodegeneration and brain atrophy are seen in one of the models (70), but the other 2 mutants await more detailed neuropathological characterization.

Taken together, the symptoms and relative severity of disease in these NCL mouse models mice are generally consistent with the corresponding form of human NCL. Behavioural studies show that all the mice have NCL subtype-appropriate degrees of progressive psychomotor abnormalities and deficits which are demonstrated by poor learning and memory skills, and a progressive motor disorder characterised by tremor, ataxia and spasticity. These mice develop a variety of seizure types, sometimes causing premature death. Over time, the NCL mice become progressively more immobile, with a lack of grooming and consequent dermatitis, followed finally by complete paralysis in the earlier onset forms.

However, care must be taken in making direct correlations between gene knockouts and specific gene mutations (knockins or naturally occurring) in mice and the mutation spectrum in affected patients. A significant genotype-phenotype correlation has been established for the human NCLs, whereby less deleterious mutations can result in milder and protracted forms of disease (17, 59, 69, 71, 100). Furthermore, differences in the genetic background have a clear influence on NCL mouse phenotype (68), and it is important to note that the majority of these mouse models have only been analysed on a mixed strain background. As such, efforts are currently underway to breed NCL mice onto a uniform genetic background to facilitate future characterization of these mice by a variety of morphological, behavioural and functional genomic techniques.

Other related mouse models. Recent studies have defined phenotypes that resemble

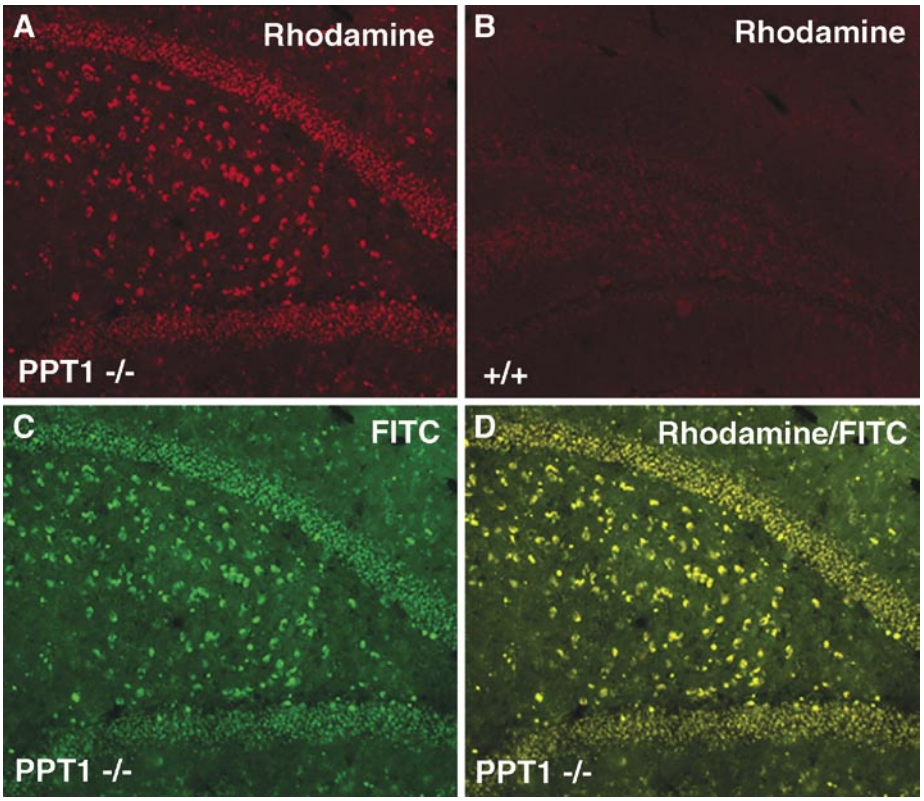

Figure 1. *Accumulation of autofluorescent storage material in murine NCL.* Unstained sections through the hippocampal formation of 7-month-old PPT1 null mutant mice (**A**, **C**, **D**) viewed by epifluorescence using rhodamine (**A**) and FITC (**C**) filter sets (merged in **D**), reveal the prominent intracellular accumulation of autofluorescent storage material within neurons in the hilus and dentate gyrus in this model of infantile NCL. In comparison, sections from age matched controls (**B**), contain only few scattered deposits of storage material.

NCL in mice carrying null mutations in genes not previously connected with this group of disorders (Table 3). Although their phenotypes are at odds with the known pattern of NCL presentation (Table 4), these mice are of great interest for their potential to provide new insights into lysosome function and NCL disease pathology. Moreover, it is possible they may represent new genes associated with one of the, as yet unidentified, forms of human NCL.

Gene-targeted knock-out mice with mutations in two lysosomal proteases, cathepsin D and palmitoyl protein thioesterase 2 (PPT2), have phenotypes that are most consistent with early-onset forms of NCL (30, 31, 50, 52, 83). PPT2 is a thioesterase related to the infantile NCL protein PPT1. PPT2 deficient mice display a similar but less aggressive neurodegenerative course compared to PPT1 null mutant mice (30), with marked extraneural features which are not seen in other NCL mice, but are more common to other lysosomal storage disorders (31). Cathepsin D deficient mice have a more severe neurological phenotype visible from 2 weeks of age, which is characterized by blindness and frequent seizures (50, 52, 83). These mice also have a visceral disorder caused by severe intestinal necrosis resulting in anorexia, and do not survive beyond 27 days (83). Although the exact time course of CNS neuropathology in cathepsin D null mutant mice awaits complete characterisation, cathepsin D deficiency has also been presented as a novel NCL form in sheep which display CNS cortical atrophy and early death (97).

The CLC3 gene which codes for a voltage-gated chloride ion channel has also been connected to NCL pathogenesis based on analysis of three separate knockout mouse models (19, 90, 110). Although one model was reported to exhibit intralysosomal accumulation of mitochondrial ATP synthase subunit c (110), this has not been confirmed in the other CLC3 null mutant mice. Furthermore, while CLC3 null mutants exhibit an early-onset neurodegenerative phenotype (19, 90, 110), the course is rather different from the NCLs and includes complete degeneration of the

Mouse model		CNS neuropathology			Retina	Somatic tissue pathology	Refer-ences
Gene and protein product	Design, strain and mutation	EM ultrastructure, neuronal degeneration and loss	Reactive gliosis	Brain atrophy	Retinal defects	Somatic tissue defects	
Cathepsin D; CTSD (Lysosomal aspartyl protease)	Knock-out, Mixed C57BL/6J-129/Sv. Neo gene replaces exon 4	Autophagic membraneous structures with unstructured granular contents and some fingerprint profiles. Degeneration and shrinkage of neurons in various CNS regions			Retinal inclusions including fingerprints at P23. Behaviour tests indicate blind at P23	Progressive intestinal atrophy from 3 wks, usually eventual widespread intestinal necrosis and thromboemboli. Destruction of thymus and spleen with lymphoid cell loss. ATP synthase subunit c-positive inclusions in liver,	(50, 52, 83)
PPT2; Palmitoyl protein thioesterase 2	Knock-out, Mixed C57BL/6J-129/Sv. Neo gene replaces exon 3	Multilamellar membrane profiles from 15 mo. TUNEL-positive cell death in cortex, thalamus, hippocampus. Moderately decreased dendritic arborisation in granule cells	Mild increase in GFAP staining in areas of CNS cell death	Brain weight decreased by 10% at 15 mo. Cortical, cerebellar atrophy	Autofluorescent storage material in retinal cells	Widespread autofluorescent accumulation and clear visceral effects: Splenomegaly, foamy spleen histiocytes. Pancreas enlarged and lipofuscin-pigmented. Occasional hepatomegaly. Bone marrow infiltration by macrophages .	(30, 31)
CLCN3. CLCN3 (CLC-3 chloride channel)	Knock-out, Mixed C57BL/6J-129/Sv. Neo gene replaces exon 3	No storage material apparent. Complete loss of the hippocampus due to degeneration and cell death from 2 wks	Activation of hippocampal microglia at P21	Drastically reduced brain size after 3 mo. with absent hippocampus	Post natal retinal degeneration commences at P12, blind due to complete photoreceptor loss by P28.		(90)
CLCN3. CLCN3 (CLC-3 chloride channel)	Knock-out, Mixed C57BL/6J-129/Sv. Neo gene replaces exon 5-6	Heterogeneous unstructured lipofuscin deposits in hippocampus at 6 wks. Loss of hippocampal pyramidal cells	Membraneous autophagic vesicles in astrocytes	Atrophic hippocampus by 2 mo. and enlarged lateral ventricles	Outer and inner segments and outer nuclear layer lost by 6 weeks. Blindness suggested by behaviour	Small intestine atrophied and necrotic by 6 weeks, loss of ileal mucosa. Mice that die 3-4 wks after weaning have necrosis of whole intestine. ATP synthase subunit c storage in liver	(110)
CLCN3. CLCN3 (CLC-3 chloride channel)	Knock-out, Mixed C57BL/6J-129/Sv. Neo gene replaces 13 bp. of exon 6 and all exon 7.	Profound progressive loss of hippocampal neurons from anterior to posterior. GABAergic GAD-positive cells. Cell degeneration/loss in entorhinal cortex	Marked astroglia activation, GFAP staining	Hippocampus normal at P23, progressive atrophy and total loss of hippocampus by 12 mo. Thinned entorhinal cortex	Rapid progressive postnatal photoreceptor degeneration, complete cell loss by P23. Similar course to (90)		(19)

Table 3.

hippocampal formation that is not a feature of the NCLs.

Large animal models of NCL. As comprehensively reviewed elsewhere (46, 49, 63), an NCL-like phenotype has been described in a number of larger domestic animal species including canine (45, 53, 65, 75, 93), ovine (12, 43, 44, 73, 92), equine (99) and bovine (36, 66) species. These large animal models offer significant advantages over their murine counterparts, with a more complex CNS and a prolonged time course to study disease progression. The opportunity to study the biodistribution and penetration of therapeutic molecules over time within a CNS of comparable size to that of human patients should be very valuable for extrapolating these treatments to a clinical setting. With advances in the molecular genetics of human NCL, and increasingly with a view towards therapeutic approaches, it will be of growing importance to focus on models that have a defined genetic defect.

Although many of these large animal models resemble the NCLs neuropathologically, in the majority of instances the underlying gene defect has not yet been identified. The South Hampshire sheep represents the best understood large animal NCL model (41, 47, 73), is orthologous to *CLN6* and is also probably allelic to the Australian Merino sheep (12). In only one large animal model, the Swedish landrace sheep, has the genetic defect been defined (98). These sheep carry a missense allele of cathepsin D (D293N) from which a stable but inactivated form of the enzyme is expressed. This mutation gives rise to a severe congenital form of NCL with extreme atrophy of the cerebral cortex due to massive neuronal loss, and an early death (98). The genes responsible for disease in the English setter dog (62; Lingaas, personal communication) and the Devon cow (Tammen, personal communication) are expected to be isolated in the near future and similar mapping work is in progress for the Border Collie dog (Wilton, personal communication). Since none of these genes appear to be allelic with the known human NCL genes, these models may represent novel NCL loci.

Mouse model	Neurological behavioural disorder			References
Gene and protein product	General	Motor	Seizures	
Cathepsin D; CTSD (Lysosomal aspartyl protease)	Develop normally during the first 2 weeks, stop thriving in the third week and die in a state of anorexia due to intestinal necrosis and reduced feeding at P25-27	Tremor, ataxia from P20.	Repetitive seizures from P20. Some mice die from tonic seizure/respiratory arrest	(50, 52, 83)
PPT2; PPT2 (Palmitoyl protein thioesterase 2)	Viable, fertile. Increased abdominal girth due to visceral defects. Mortality increased, 50% survival at 11 mo and 10% at 17 mo.	Ataxic gait after clasping phenotype appears. Clasping phenotype from 29 wks. 50% by 9 mo., 100% by 13 mo.	Frequent myoclonic jerks without spontaneous seizures	(30, 31)
CLCN3. CLCN3 (CLC-3 chloride channel)	Become smaller than littermates soon after birth. Stereotypic circling behaviour. Overall higher mortality but most survive over 12 mo.	Open field showed increased locomotor hyperactivity. Rotarod deficits due to tremor, improved with learning.		(90)
CLCN3. CLCN3 (CLC-3 chloride channel)	Growth deficit soon after birth, 25% reduced body weight by 6 weeks with kyphoscoliosis. 30% die after weaning, rest can survive at least to 7 mo.	Rotarod deficits, hyperlocomotive activity at 6 wks. Hanging wire test deficits at 6 wks		(110)
CLCN3. CLCN3 (CLC-3 chloride channel)	Growth impaired from 1 mo. Hyperactive from 2-3 wks, kyphoscoliosis. High mortality, constant death rate over first 10 mo, peaks during weaning. 50% survival by 6 mo.	Waddling gait from 2-3 wks. No evidence of a myopathy. Clasping phenotype in most mice.	Occasional generalized tonic-clonic seizures seen in adults , can cause death	(19)

Table 4.

SELECTIVITY OF CELL DEATH IN THE NCLs

Neuronal versus somatic cell loss? Since the NCLs are progressive neurodegenerative disorders, the majority of pathological studies have focused, unsurprisingly, upon morphological changes within the CNS of affected patients (35). Since the initial presentation of many affected children is with visual impairment and subsequent neurological decline, the retina and brain have been studied in more detail than the visceral organs (35, 38). Although accumulation of autofluorescent storage material is apparently common to virtually all cell types in the body (38), it has always been assumed that the effects of disease are confined to the CNS. Such preferential targeting of the CNS may arise from the fact that neurons are post-mitotic and there is very limited capacity for renewal of dysfunctional or dying neurons.

Alternatively, neuron-specific roles for several CLN genes have been suggested, which may be critical for neuronal survival, but are less important for cellular viability outside the CNS (1, 60, 64). Indeed, given the highly polarized nature of most neurons, reported disturbances in the trafficking of mutated CLN gene products are likely to have more severe consequences for neuronal function than in somatic cells (64, 78). Another plausible explanation for relative neuronal vulnerability is the existence of other proteins or enzymes that can compensate for the missing gene product outside the CNS. For example in LINCL, the function of missing TPP1 can be compensated in somatic tissue by another related lysosomal protease DPP1 (cathepsin C) that is apparently not expressed in CNS tissues (2).

Paradoxically, expression of the CLN proteins is often very low within the CNS, where degenerative effects are most pronounced. Indeed, it is apparent that there is no direct relationship between the normal physiological expression levels of these proteins and the extent of subsequent pathological change. For example, in LINCL despite the ubiquitous distribution of TPP1 (51), the deleterious effects of mutations apparently manifest only in neuronal cells. In $CLN3^{\Delta ex7/8}$ knock-in mice, the relative expression levels of the truncated CLN3 protein also bear no relation to subsequent degenerative events (16). Despite a higher expression of truncated CLN3 protein in the liver compared to brain in these mice, there is no evidence of liver dysfunction in these animals, whereas retinal cells that express very little truncated CLN3 protein are markedly affected (16).

Although each of the CLN gene products is expressed widely outside the CNS, it cannot be assumed that somatic and visceral organs are completely unaffected in these disorders. PPT2 null mutant mice that display a mild NCL-like phenotype have recently been shown to exhibit significant extraneuronal pathology including splenomegaly with bone marrow infiltration by nonfoamy macrophages and multinucleated giant cells (31). These data emphasize the importance of building a detailed pathological history in NCL models throughout the body rather than concentrating solely upon the CNS. Indeed, if CNS directed therapeutic approaches ultimately prove effective, it is probable that extraneuronal roles for the CLN proteins may be unmasked.

Selectivity of pathological effects within the CNS. *Regional and laminar effects.* In the NCLs, as in many neurodegenerative disorders, there is widespread neuronal loss by the later stages of disease, accompanied by profound glial activation (35, 38). The degree of neuronal loss varies between different types, being most profound in the earlier onset forms (5, 6, 32, 33, 37, 96). In contrast to the devastating loss of cortical neurons and resulting cortical atrophy, subcortical structures are apparently relatively

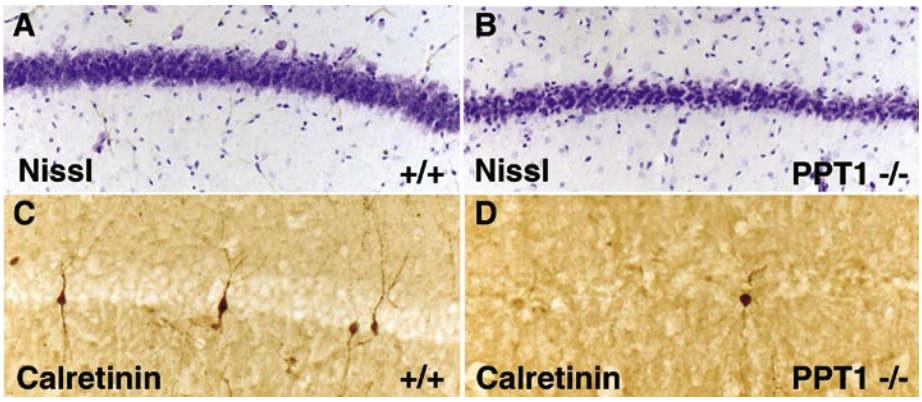

Figure 2. *Loss of hippocampal neurons in murine NCL.* Coronal sections through the hippocampus of 7-month-old PPT1 -/- and age matched controls (+/+) either Nissl stained (**A, B**) or immunohistochemically stained for the interneuron marker calretinin (**C, D**). In this example the CA1 subfield in PPT1 -/- is severely thinned (**B**) and also displays a significant loss of calbindin-positive interneurons (**D**), together with abnormal dendritic morphology in the few persisting interneurons.

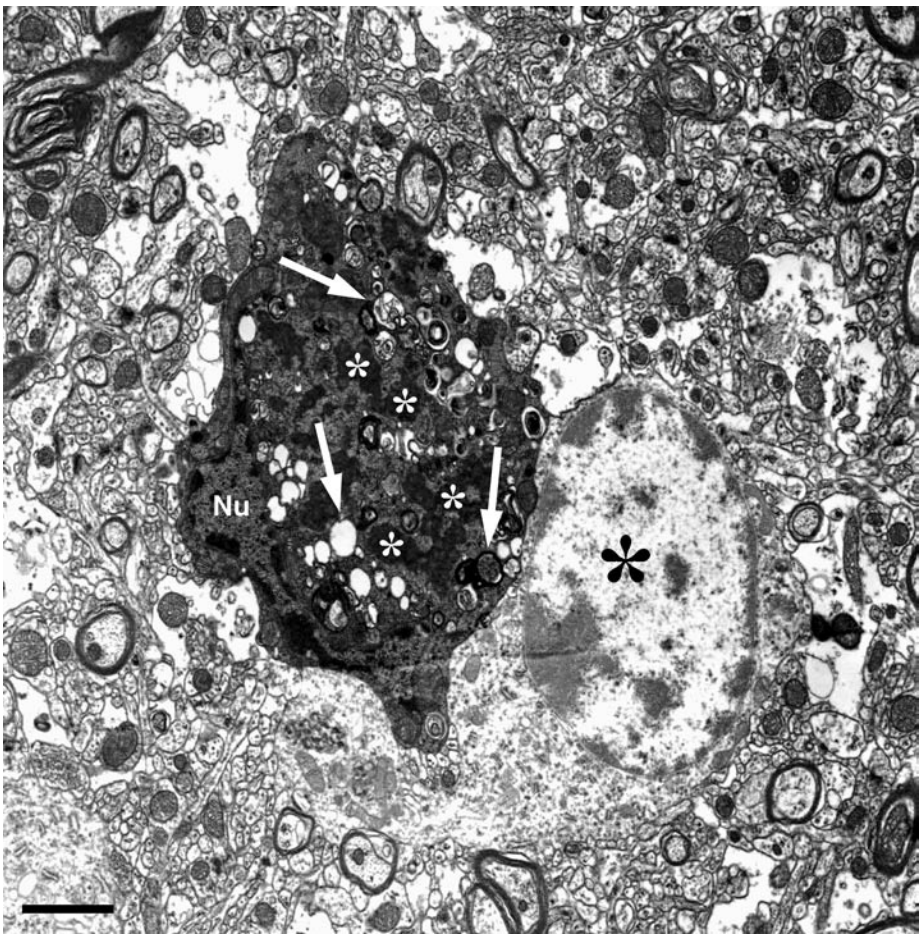

Figure 3. *Autophagic neurodegeneration in murine JNCL.* Electron micrograph from the cortex of a 12 month old CLN3 null mutant mouse reveals a degenerating cortical neuron with electron dense cytoplasm, containing prominent accumulations of storage material (white *) and typical autophagic vacuoles with a range of morphologies (arrows). This neuron is in close contact with a reactive astrocyte (black *) and retains an intact nucleus (Nu), with none of the ultrastructural features of apoptosis or necrosis. Scale bar = 2 µm. Electron micrograph kindly provided by Mark Turmaine, UCL.

spared, but this has not been characterized in detail. Pronounced cerebellar loss is a feature in human NCL, particularly in INCL (32, 33), and its variant CLN5 (96).

As already discussed, making comparisons between NCL mice is complicated by having a range of strain backgrounds, but progressive cortical atrophy is a feature of all characterized mouse models of NCL (13, 16, 30, 48, 56, 70), and is also evident in large animal models (73; Oswald, Cooper and Palmer, unpublished observations). It is now apparent that cortical atrophy is not uniform across the cortical mantle, but is more pronounced in areas with sensory versus motor function and with lamina specific events in more than one species. As would be expected, cortical atrophy is particularly advanced in severely affected PPT1 null mutant mice (30, 54), but surprisingly these mice also show evidence for subcortical atrophy (Bible, Gupta, Hofmann and Cooper, unpublished observations). In contrast to patient derived material, mouse models show little evidence for cerebellar atrophy. This discrepancy may simply reflect that mouse models do not live long enough for cerebellar atrophy to become apparent, although more subtle pathological changes are evident in severely affected mice (30).

Cell type specific effects. Selective neuronal vulnerability is a common feature of many neurodegenerative disorders and the NCLs are no exception, with pronounced loss of GABAergic interneurons initially reported in both human tissue and canine models (105). The involvement of GABAergic neurons in lysosomal storage disorders (LSDs) has been proposed previously (103) and specifically is suggested to occur in non-murine models of NCL (65, 73). Previous studies of the neocortex of human NCL patients described a loss of small stellate neurons, which were presumed to be GABAergic (5, 6), and a loss of ultrastructurally identified inhibitory synapses (107). Golgi studies revealed that many cell types exhibit the formation of meganeurites and less dendritic spines (6, 104), but exhibit a different type of enlargement from that which characteristically affects GABAergic neurons in other storage disorders (102). GABAergic neurons are thought to exhibit higher metabolic rates and have greater oxygen demand and firing rates than other neurons (40, 103) that might render them more vulnerable in the NCLs. More recently the presence of an autoantibody to GAD65, the enzyme necessary for the synthesis of GABA, has been reported in *CLN3*$^{-/-}$ mice (10) and JNCL patients (10, 11). This raises the possibility of an autoimmune component to this selective loss of interneurons, although GAD65 autoantibodies are apparently not present

in other forms of NCL (Pearce, personal communication).

Several reports now confirm the loss of GABAergic interneurons in animal models of NCL (13, 14, 56, 70, 73) (Figure 2), and in autopsy material from different forms of human NCL (Tyynelä, Cooper and Haltia, unpublished observations). Mouse models of CLN1, CLN3, CLN6 and CLN8 each exhibit complex patterns of interneuron loss within the hippocampus and cortex (13, 14, 56, 70, 73), although the extent and timing of interneuron loss varies markedly among different models (15). It is now apparent that interneuron subpopulations that express a range of calcium binding proteins or neuropeptides exhibit different degrees of vulnerability, patterns that are also retained in ovine and human NCL (73; Tyynelä, Cooper and Haltia, unpublished observations). Although these differences may reflect the relative buffering ability of individual calcium binding proteins (20), neuronal populations in the amygdala that are immunoreactive for the same calcium binding proteins and neuropeptide antigens are not significantly affected even in aged *Cln3*$^{-/-}$ (Chakrabarti, Pearce and Cooper, unpublished observations).

Different forms of human NCL share a consistent pattern of neuronal degeneration in the hippocampus with heavy involvement of CA2-CA4 but relative sparing of CA1 (34), which is distinct from neuronal loss in temporal lobe epilepsy. This pattern of pyramidal cell loss is closely mirrored by subpopulations of interneurons in human NCL (Tyynelä, Cooper and Haltia, unpublished observations), and murine NCL where distinct subfield specific interneuron loss is also evident. Interestingly, a similar pattern of neuronal loss is evident in a CLC3 null mutant mouse reported to exhibit NCL-like pathology (19). As such, it appears that the cues which determine neuronal survival are complex and depend upon precise cellular location rather than solely phenotypic identity. Given the complex heterogeneity of cell populations within the CNS, a detailed understanding of the response of each cell type to disease will ultimately be required.

Specific events within glial cell populations. There is considerable evidence for the activation of glial cell populations at the end stage of all forms of NCL (27, 33, 35, 91). Reactive gliosis has also been observed in animal models of NCL (Table 1). Such glial activation is common to many neurodegenerative disorders, and during disease progression reactive astrocytes and microglia may serve as early indicators of damage within affected areas (24, 55, 89). Until recently, little was known regarding glial activation and the neuroimmune response in relation to neuronal loss in the NCLs. Autopsy material derived from individuals with different forms of NCL shows a consistent and regionally specific pattern of astrocytosis and microglial activation (Tyynelä, Cooper and Haltia, unpublished observations). Similar data can be obtained from null mutant mouse models, with highly restricted microglial activation and more widespread astrocytosis (15). Significantly, these glial responses are evident in presymptomatic animals many months before obvious neuronal pathology. The underlying molecular cues are presently unknown and it remains to be demonstrated whether these events represent degenerative or regenerative responses.

CELL DEATH MECHANISMS IN NCL

It is not known how defects in up to 8 different genes result in the similar neurodegenerative phenotype of NCL, albeit with widely variable onset and rates of progression. The precise cues that activate cell death are poorly understood, and it is not clear whether the build-up of storage material is directly associated with neuronal cell death. Neuronal cell death can occur by at least 3 diverse mechanisms: apoptosis, autophagy and necrosis (111). Understanding which mechanisms operate in different forms of NCL may have therapeutic implications, and these events are now the focus of studies in human tissue and animal models of NCL.

Programmed apoptotic cell death, which is regulated by a well conserved set of molecules consisting of the caspase and Bcl-2 family and Apaf-1 (111), seems to be the main mechanism of retinal cell death in mouse models of retinitis pigmentosa (94). Studies in NCL mouse models also support apoptosis as the main cell death mechanism in the retina (16, 52, 87). Apoptotic neuronal cells were detected in human LINCL and JNCL CNS tissue, and in South Hampshire sheep using the terminal dUDP nick end-labelling (TUNEL) staining method, flow cytometry and electron microscopy (57). TUNEL positive cells were also seen in the cerebellum of PPT1 deficient mice (30). A role for CLN3 in a novel antiapoptotic pathway has also been suggested (81), and localized to certain CLN3 protein motifs in cell culture systems (79).

Although apoptosis has been described in several forms of NCL, other mechanisms also appear to operate. Indeed, in nclf and juvenile NCL mouse models no morphological evidence exists for an apoptotic mechanism of cell death (8, 16). Furthermore, the ultrastructural characteristics of cell death in CLN3 null mutant mice are autophagic in nature (Figure 3), with TUNEL assays and immunohistochemistry proving negative for apoptotic features (Mitchison, unpublished data). Autophagic cell death in neurons is associated with the formation of autophagosomes, electron dense membraneous vacuoles and engulfment of entire organelles (58, 80). Recent studies in the *cathepsin D* deficient mouse have also raised the possibility of autophagic mechanisms as targets for disruption in the ceroid lipofuscinoses, as has also been suggested for other forms of neurodegeneration (50, 80).

The debate over which cell death mechanisms operate in the NCLs is mirrored by discussions in other neurodegenerative conditions (28, 111). Caspase-independent programmed cell death mechanisms have also recently come to the fore (28, 61). Furthermore, it has been shown that autophagic and apoptotic processes can occur simultaneously or sequentially in degrading neurons, and the mechanisms of degeneration may also differ in different compartments of the cell (21, 109). It is increasingly likely that, as in other neurodegenerative disorders, a spectrum of cell death events takes place in the NCLs, with considerable blurring of categories (28). Indeed, these events may vary between subtypes of NCL and in different brain regions or cell types and it will require detailed investigations to resolve this issue.

UNDERSTANDING PATHOGENESIS—THE CLINCAL IMPLICATIONS?

Detailed characterization of animal models is providing invaluable information about the earliest stages of pathogenesis and its progressive nature that have direct

clinical implications. These data are helping to identify targets that can subsequently be linked to surrogate markers (eg, neuroimaging with spectral analysis) and potentially be applied as non-invasive measures in human subjects. In this manner, clinicians will be better equipped to monitor disease progression in patients and to judge therapeutic efficacy of approaches that may ultimately reach clinical trials. Better understanding of cell death mechanisms in the NCLs may also provide avenues to devise novel therapeutic strategies. However, before these clinical goals can be realised, it is paramount that candidate strategies are rigorously tested in appropriate animal models that have been thoroughly studied. Although many gaps still exist in our understanding of NCL pathogenesis, these animal models will prove a crucial resource in making further progress.

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