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Review

NCL disease mechanisms ☆

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ABSTRACT

Despite the identification of a large number of disease-causing genes in recent years, it is still unclear what disease mechanisms operate in the neuronal ceroid lipofuscinoses (NCLs, Batten disease). As a group they are defined by the specific accumulation of protein, either subunit c of mitochondrial ATP synthase or SAPs A and D in lysosome-derived organelles, and regionally specific neurodegeneration. Evidence from biochemical and cell biology studies indicates related lesions in intracellular vesicle trafficking and lysosomal function. There is also extensive immunohistological evidence of a causative role of disease associated neuroinflammation. However the nature of these lesions is not clear nor is it clear why they lead to the defining pathology. Several different theories have proposed a range of potential mechanisms, but it remains to be determined which are central to pathogenesis, and whether there is a mechanism consistent across the group, or if it differs between disease forms. This review summarises the evidence that is currently available and the progress that has been made in understanding these profoundly disabling disorders. This article is part of a Special Issue entitled: The Neuronal Ceroid Lipofuscinoses or Batten Disease.

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1. Introduction

The neuronal ceroid lipofuscinoses (NCLs, Batten disease) are devastating neurodegenerative diseases of children, leading to severe mental and physical decline, and finally death. These diseases were initially grouped together because of their clinical similarities and broadly uniform neuropathological features, which include a similar progression of clinical symptoms, lysosomal accumulation of fluorescent storage material, with defining histochemical properties and ultrastructures, as well as profound neurodegeneration and widespread gliosis within the CNS.

Despite the identification of several disease causing genes and many studies of the respective NCL proteins, the underlying pathological mechanisms remain obscure. Even the fundamental question as to whether there is a common pathway that unites all or some of the NCLs remains unanswered.

A number of hypotheses have arisen over the years to explain NCL disease progression. Until recently its downstream pathology was considered to be a consequence of storage body accumulation, but this idea is no longer tenable. Furthermore, it has become apparent that no single level of investigation will resolve all the issues of NCL pathogenesis, but when the evidence from multiple disciplines is viewed collectively, it becomes apparent that considerable progress has been made towards understanding disease mechanisms.

2. Genetic considerations

Although it had been known for a long time that the diseases which became known as the NCLs were genetically inherited, it was not until the 1990s that any disease-causing genes were identified, and it was unequivocally established that these were actually a group of genetically distinct diseases. Since then there has been a comprehensive increase in our knowledge of NCL genetics, starting with the discovery of mutations underling classic forms, causing what are now regarded as CLN1 and CLN3 diseases, closely followed by CLN2 disease [1–3] and subsequently followed by many others. It is now apparent that these diseases occur far more widely than was originally thought and many previous estimates of incidence are far too low. Aside from the addition of *CLN5*, *CLN6*, *CLN7*, *CLN8* and *CLN10* to the NCL family, a number of other genetic forms have been proposed recently, some with more secure foundations than others. At the time of writing these include three adult onset forms, *CLN4/DNAJC5*, *CLN11/GRN*, *CLN13/CTSF* [4–6], another infantile form *CLN14/KCTD7* [7], and another juvenile form, *CLN12/ATP13A2* [8].

While diagnoses of adult onset NCL have a strong historical basis on clinical and neuropathological grounds and there are at least ultrastructural similarities in the nature of the stored material, information

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about other common differential classification markers is not complete with these later additions, and none meet the full suite of classification criteria. Furthermore mutations in some of the genes implicated are associated with other diseases, including a frontotemporal lobar degeneration in people heterozygous for mutations in *CLN11/GRN* [5] while mutations in *ATP13A2* also cause a rare parkinsonism, Kufor–Rakeb syndrome [8]. A stated above little is known of the underlying pathogenic mechanisms that may unite the NCLs, so it follows that a definitive declaration of which diseases should, and which should not, be grouped together in the NCLs is also not possible. Nevertheless abundant evidence of defining similarities at the biochemical, cell biology, and neuropathological levels indicates where it should be productive to look for common steps in the disease mechanisms, as reviewed below. Meanwhile the addition of more recent forms in the catalogue of NCLs should be treated somewhat tentatively, relative to the collective identity of the classic forms. At some point there will be a need for a reconsideration of the requirements for a disease to be delineated as an NCL, for the general classification to be useful. Meanwhile it is interesting how little commonality can be inferred from the diverse nature of the causative genes, shown in comprehensive reviews of the genetic bases of these disorders [9,10].

3. Biochemical investigations

3.1. Storage body analyses

The last century saw the common assumption of a connection between the material stored in lysosomal storage diseases and the disease pathogenesis. Until the 1960s the disorders that are now termed the NCLs were usually grouped together with some other lysosomal storage diseases under a catch-all description of “amaurotic family (familial) idiocies”. Two lines of enquiry led to the NCLs being considered as a separate category. Biochemical analyses of the material stored in the other amaurotic family idiocies proved to be very informative. For a number of diseases postulates of missing enzyme activities, derived from the structures of the stored material, were confirmed when the enzyme activities were found in control individuals but not in patients. A number of lysosomal enzymes with acidic pH optima were discovered this way, but this technique was unrewarding for what became the NCLs. The second distinction was made on the basis of the now characteristic histopathological and ultrastructural properties of the storage bodies, which also indicated that the NCLs were a separate group of diseases [11–13]. A historical perspective on these diseases and a detailed description of storage body ultrastructures are provided by other authors in this special issue.

Because the storage bodies resembled the so-called lipopigments ceroid and lipofuscin, an analogous argument for their origin was inferred; that they arose from uncontrolled lipid peroxidation, leading to the generation of reactive aldehydes assumed to cross link with the amino groups of proteins, lipids and nucleic acids forming an insoluble intractable fluorescent complex. Although no such complex has ever been characterised, these names have stuck and connected thoughts that they might drive pathogenesis persist to this day.

Simple methods of isolating and characterising the storage bodies were developed in studies on the CLN6 ovine model. Storage body preparations from a number of tissues were found to contain around 2/3 of protein, subsequently shown to be the c subunit of F_1F_0 ATP synthase complex (subunit c), the rest being mainly lipids, with a profile attributable to a lysosomal/endosomal origin, including lysobisphosphatidic acid, also called bis(monoacylglycero)phosphate [14–17]. No sign of an intrinsic fluorophore has been found among the components of these isolates. Furthermore the fluorescence has been reconstructed by reconstitution of storage body-like structures from non-fluorescent subunit c and sheep liver phospholipids, confirming that this is an aggregate, not an intrinsic property [18,19]. Since then, subunit c has been identified as the main storage protein in most forms of NCL, including CLN2 disease, CLN3 disease, CLN5 disease, CLN7 disease, and CLN8 disease [20–25]. Subunit c storage is generalised in these diseases, this protein being the major component of storage bodies in all tissues that contain them, including liver, kidney and pancreas. It has also been confirmed that the structure of the stored subunit c is identical to that mature protein functional in intact mitochondria, including the trimethylation of lysine 43 [26], so this is not the storage of a partial degradation product of some proteolytic pathway. The N-terminal mitochondrial import signalling sequences have been removed, indicating a history of importation into the 16 oligomer multimeric ATPase complex in the inner mitochondrial membrane. Subunit c is packed into a ring of eight c subunits that drives the rotation of the γ subunit forcing conformational changes in the F_1 portion of the molecule and synthesis of ATP [27]. None of the other ATP synthase subunits are stored [28], highlighting the specificity of subunit c storage and its turnover pathway.

Different proteins are stored in CLN1 disease, CLN4 disease (Parry disease) and CLN10 disease, as well as in miniature Schnauzer dogs affected by NCL, where the storage of the sphingolipid activator proteins (SAPs) A and D has been reported [26,29–31]. Some subunit c storage has also been reported in cathepsin D (CTSD, CLN10) deficiency [32]. Knowledge of the storage material and sites of storage in the more recently classified forms of NCL is variable, although the ultrastructures and fluorescent properties of the neuronal storage bodies are consistent with being NCLs. In a sense the classic forms of NCL are misnamed, the phrase ceroid lipofuscinosis being misleading because the storage bodies do not have the molecular composition usually attributed to ceroid or lipofuscin, as explained above. Whether or not they should be called neuronal is also a moot point. On one hand the neurological symptoms and neurodegeneration indicate a neuronal disease, but on the other hand storage body accumulation is not exclusively neuronal but generalised, with storage bodies accumulating in most cell types throughout the body.

Nothing is reported of the nature of the molecular composition of the stored material, or of non-neuronal storage, in the novel adult forms, *CLN4/DNAJC5* [4], *CLN11/GRN* [5] or *CLN13/CTSF* [6]. There is evidence for subunit c storage in neuronal tissue and lymphoblasts in the novel infantile form *CLN14/KCTD7* [7], while some uncharacterised non-neuronal storage is indicated in the novel juvenile form, *CLN12/ATP13A2* [8]. Some accumulation of subunit c has also been noted in a number of other lysosomal storage diseases, including the mucopolysaccharidoses (MPS) [33,34], recently well described in MPS III B [35]. However this accumulation is restricted to some cells in specific areas of the brain while other material accumulates in other cells, a very different circumstance to the specific and generalised storage of subunit c in lysosome derived bodies characteristic of the major forms of NCL described above.

3.2. Functions of the gene products

Identification of the protein lesions responsible for these NCLs gave no immediate insight into why subunit c or SAPs A and D should be specifically stored, nor did it provide any direct clues about the pathogenic cascade. CLN1 (PPT1), CLN2 (TPP1) and CLN10 (CTSD) have been identified as bona fide soluble lysosomal enzymes. The catalytic mechanism of protein palmitoyl protein thioesterase (PPT1, CLN1) has been inferred from its 3-D structure which revealed a classical α/β serine hydrolase consisting of two major domains with a fatty acid binding groove down the centre [36]. Structural determination of the covalent fatty acyl-enzyme intermediate showed that palmitate is bound in an extended conformation along a hydrophobic groove in the second domain of PPT1. Metabolic labelling studies showed that the enzyme removes fatty acids from fatty acylated proteins in cultured cells [37–39].

Recombinant tripeptidyl peptidase 1 (TPP1, the CLN2 protein) is efficiently delivered to the lysosomes by mannose 6-phosphate-receptor-mediated endocytosis in CLN2 affected fibroblasts, which restores normal enzyme activity and ameliorates the accumulation of mitochondrial ATP synthase subunit c [40–42]. Its wider *in vitro* substrate specificity has been explored using combinational peptide libraries [43]. The CLN10

protein (cathepsin D, CTSD), is a classical lysosomal aspartic protease with the active site cleft located between two lobes, each lobe contributing an aspartic acid residue to the catalytic centre [44]. Several proteins function as substrates of CTSD *in vitro*, but the *in vivo* substrates are still unknown. The presence of mannose-6-phosphate residues on the CLN5 protein, that interact with the lysosome targeting mannose 6 phosphate receptors, indicates that it is a soluble lysosomal protein [45–47], but its function is still known.

The other gene products are all membrane bound proteins of unknown function which have been tentatively localised to a number of subcellular sites, residing in the lysosomal membrane or in compartments implicated in pre-lysosomal processing. The CLN3 protein is probably a lysosomal or endosomal membrane protein [48–50], the CLN7 protein a membrane protein [51], the CLN8 protein an endoplasmic reticulum–Golgi resident [52], and the CLN6 protein an endoplasmic reticulum resident [53–55].

Why deficiencies in these gene products result in similar clinical, pathological and biochemical changes is a long-standing question. One option is that the NCL proteins may participate in a common biological pathway, in which shared protein associations may play an important role. At a biochemical level this is certainly true for CLN2 disease, CLN3 disease, CLN5 disease, CLN6 disease, CLN7 disease and CLN8 disease where subunit c storage is definitive and specific, suggesting a series of related lesions in a turnover pathway [24]. However apart from experiments showing that subunit c degradation is inhibited by the absence of gene products for *CLN2* [40–42], nothing definitive is known about the mechanism of the dissociation of subunit c molecules from the oligomeric ATP synthase complex and their turnover.

Much has been inferred about the structures of the CLN2, CLN3, CLN5, CLN6, CLN7 and CLN8 proteins from computer-generated predications of monomeric structures of these gene products. These inferences should be treated with caution, not least because stand-alone membrane bound proteins are a rarity, start sites for translation are sometimes confused, and little is known of the post-translational processing of these molecules.

Possible protein–protein interactions between *PPT1*, *TPP1* and *CLN3* encoded proteins were examined in a yeast two hybrid model study, which provided no evidence that they interact with each other [56]. However a co-immunoprecipitation study of over-expressed proteins in COS-1 cells reported interactions between the CLN2 and CLN3 proteins with the CLN5 protein, but not with the CLN1 enzyme [57]. More recent overexpressed recombinant protein studies reported interactions between the *CLN5* and the *CLN1*(*PPT1*), *CLN2(TPP1)*, *CLN3*, *CLN6* and *CLN8* gene products [58]. However, the CLN5 protein used in these studies was fused with GST and expressed in *Escherichia coli*, raising questions about the reported protein–protein interactions. It is likely that expressing a highly modified protein such as CLN5 in *E. coli* does not produce a native protein because proper glycosylation, cleavage of the N-terminal sequence, and correct folding of the protein require ER-processing. Furthermore the recombinant protein was made using initiation from an incorrect ATG start site. Over-expression of CLN (or any other) proteins, can affect their structure, resulting in interactions that would not occur *in vivo*. Others have also studied the transport and maturation of CLN5 protein [59–61].

There are other technical problems with these sorts of studies. The purity of the proteins utilised and aggregation of hydrophobic proteins are issues. Since cell extraction eliminates the spatial constraints within cells that limit protein–protein interactions, it can result in interactions not possible within intact cells. Lack of reliable antibodies is also a problem, and antibody specificities are not validated or verified in most studies, causing further uncertainty.

3.3. Secondary biochemical defects

Other biochemical investigations include reports of deficits in oxidative phosphorylation, which have not been substantiated, principal component analysis of the neuroactive amino acid concentrations in mouse and sheep models and analysis of circulating antibodies. Excitotoxicity was postulated to be involved in pathology of the NCLs some time ago [62,63], and could represent a therapeutic target [64].

Populations of γ-aminobutyric acid (GABA)ergic interneurons have been shown to be affected in human, sheep and mouse NCLs [65–69]. A regional and time dependent decline in γ-aminobutyric acid (GABA) concentrations was notable among a number of changes revealed in metabolomic studies of Cln3 affected mice and CLN6 affected sheep brains [70,71]. These were consistent with glial cell activation and neurodegeneration, beginning in the frontal and occipital lobes, in agreement with histopathological data (see below). Changes in glutamate and glutamine concentrations were also detected in mouse and sheep cerebrospinal fluids (CSFs). However, these changes occurred after clinical disease had become apparent, indicating that any changes in glutamate/glutamine cycling occur as a consequence of the primary deficits, and thus that excitotoxicity is unlikely to be their cause.

A comparison of CSF neuropeptide concentrations in patients with the classic juvenile CLN3 disease, classic late infantile CLN2 disease and neuropeptide and neuroactive amino acid concentrations in CSF from sheep with CLN6 disease revealed marked disease related increases in the concentrations of neuron specific enolase and tau protein in CLN3 disease patients, but not in a severely affected CLN2 disease patient nor in CLN6 affected sheep [72]. No changes were noted in S-100ß, glial fibrillary acidic protein (GFAP) or myelin basic protein (MBP) in patients, or of S-100ß, GFAP or insulin-like growth factor-1 (IGF-1) in affected sheep. Furthermore no disease related changes in concentrations of the neuroactive amino acids; aspartate, glutamate, serine, glutamine, glycine, taurine and GABA were observed in CSF from affected sheep. It was concluded that the changes in CLN3 disease patients may be progressive markers of neurodegeneration, or of underlying metabolic changes perhaps associated with CLN3 specific changes in neuroactive amino acids. The lack of changes in the CLN2 disease and CLN6 disease subjects indicates that these changes are not shared by the CLN2 or CLN6 forms and changes in CSF concentrations of these compounds are unreliable as general biomarkers of neurodegeneration in the NCLs.

4. Histopathological changes

4.1. Neurodegeneration

A range of animal models have proved invaluable for studying NCLs. These include colonies of animals established following the diagnosis of naturally occurring incidences of disease dogs, sheep and cattle [73,74]. Naturally occurring mouse models are complemented by others resulting from gene manipulation, to create null allele 'knockout' mice, or to recreate specific disease causing mutations in knock-in mice, reviewed in [75–78]. Typically, these models recapitulate the key features of the corresponding human NCL diseases, including progressive loss of neurons and synapses, widespread gliosis, and the accumulation of the characteristic storage bodies. Neither affected sheep nor most affected mice models exhibit spontaneous seizure activity to the same extent as affected humans, but characteristically display movement and gait abnormalities, which sometimes progresses to hind limb paralysis. While the large animals, and most of the mouse models, have a dramatically shortened lifespan, others (*e.g. Cln3* and *Cln5* mutant mice) have a near normal longevity. These models have proved to be valuable tools to study disease progression and have yielded significant information about the regional selectivity of neurodegeneration, and its relationship to other neuropathological events.

Although neuron loss is widespread by the end of the disease, recent studies have revealed the regionally selective nature of the initiation of this loss. Overt atrophy in ovine NCL is similar to that in human NCLs [68,74,79–83], but is not nearly as apparent in the mouse models

[75–78] with the exception of *Cln2/Tpp1* knockout mice [84]. Nevertheless, several examples of selective neuron loss, including differing effects upon subpopulations of cortical and hippocampal neurons, thalamic relay neurons, and Purkinje cells of the cerebellum have been documented in murine NCLs [75–78]. Similar, but more pronounced regional selectivity is apparent in both ovine and human NCL, emphasising that these more complex brains appear to be more severely affected.

It is now clear that functional neuron loss is not related to storage body accumulation, as was first thought. Most visceral and CNS cells in affected animals accumulate storage bodies, but only specific subsets of neuronal cells are functionally affected. Even within these populations there is no correlation between neurodegeneration, neuroinflammation and storage body accumulation, as was shown in studies of CLN6 affected sheep and in various mouse models [76,81,82,85]. Furthermore it is possible to clear significant amounts of this storage material without improving the disease outcome [86].

Instead studies to date have revealed that the selective loss of specific neuron populations is preceded by localised glial activation, and synaptic pathology that is most pronounced in the same brain regions. Rather than storage material accumulation, localised activation of glia is a better predictor of the distribution of subsequent neuron loss [76,81,87]. Studies in ovine CLN6 disease have shown that the sites which show early reactive changes display the most pronounced neuron loss, with selective loss of different subtypes of GABAergic interneurons in these brain regions [81,82]. This relationship also holds true in all characterised mouse models [67,69,49,51,88–93], although the nature and extent of this glial activation vary markedly between different forms of NCL. It is not clear whether this glial activation actually contributes to neuron loss or is a protective response, but as discussed below in more detail, it appears that neuroinflammation is a key part of the pathogenesis in many, if not all, forms of NCL.

A consistent finding in murine models is the particular vulnerability of the sensory thalamocortical pathways early in disease progression [76]. Once again, the localised activation of astrocytes and microglia is closely connected to disease pathogenesis, invariably preceding or accompanying neuron loss in these brain regions [67,69,88–93]. For reasons that remain unclear, the thalamus appears to be one of the first sites to be affected pathologically in most NCL mouse models, including *Cln1/Ppt1*, *Cln3*, *Cln8/mnd* and *Cln10/Ctsd* mutant mice [67,88,90,94], with subsequent cortical pathology only apparent in the corresponding cortical region. This is not the case in the *Cln5* mutant mice where cortical neuron loss precedes that in the thalamus [93]. Cortical lesions are foremost in the large animal and human NCLs, being particularly obvious early in upper layers of the occipital and somatosensory cortices [74,79,80].

The selective loss of interneuron populations, has also been reported in mouse models [65–67,52,91,95], but regional effects upon cortical and hippocampal interneurons are more pronounced in human [96] and ovine NCLs [82]. A comprehensive survey of interneuron phenotypes in CLN6 affected sheep at different stages of disease revealed selective effects upon interneuron populations that differed markedly with time and between locations within the brain. This pattern of interneuron loss follows the pattern of glial activation [82], whereas the specific loss of gonadotropin-releasing hormone (GnRH) secreting neurons of the hypothalamus is not associated with glial activation [85]. Subcortical nuclei and the cerebellum of affected CLN6 ovine brains also retain a normal appearance, even at advanced stages of disease, despite widespread storage body accumulation and glial activation. These findings indicate that cellular location and connectivity are much more important determinants of neuron survival than phenotypic identity [82], and add a physiological dimension to the pathogenic process that we will return to later.

4.2. Neuroinflammation

Over the last decade there has been a growing realisation of the central role of neuroinflammation in the pathogenesis of a number of neurological protein accumulating and lysosomal storage diseases, including the NCLs. When the association between abnormal storage of protein, neuroinflammation and neurodegeneration was first noticed, it was assumed that the inflammation arose as a reaction to the presence of the abnormally accumulating material. Subsequent studies revealed a number of circumstances when this is not the case. For example, the suppression of inflammation resulted in a delay of the clinical progress in Sandhoff disease knockout mice, suggesting a causative role for neuroinflammation in pathogenesis [97].

That neuroinflammation actually precedes neurodegeneration was established in a series of studies on the well-characterised CLN6 ovine model [74,81,98]. Immunohistochemical analyses revealed that glial activation begins prenatally, long before significant storage body accumulation or neuron loss [81,87]. Proliferating perivascular macrophages and activated astrocytes were present at 20 and 40 days before birth respectively, progressing to focal clusters of activated microglia and astrocytes in cortical regions at birth. This glial activation proceeded in a progressive, regionally specific manner, with the visual, parieto-occipital and somatosensory cortices being first and most affected, followed by activation in the primary motor and entorhinal cortices. Later neurodegeneration followed activation in the same regional and temporal order [81].

As discussed above, glial activation has also been investigated in mouse models of NCL [67,69,86–91]. In particular *Cln3* mutant mice exhibit an early low-level activation of astrocytes and microglia prior to the appearance of symptoms, but this appears to be attenuated with a failure of the normal morphological transformation of these cell types upon activation [69,91]. These data raise the possibility that the glia are themselves dysfunctional and may exacerbate neurodegeneration. Given the close functional relationship between neurons and glia, it will be important to investigate how this may be compromised in each form of NCL.

The mechanisms by which inflammation functions in neurodegenerative disease are a matter of debate. It is considered to have both favourable and detrimental consequences, but when chronically sustained may be neurotoxic [99,100]. There is evidence that glia could be actively involved in neurodegeneration or neuroprotection. Microglial cells may mediate cell death *via* free radical, reactive oxygen species (ROS), prostaglandin or cytokine production [101,102] or be neuroprotective by scavenging free radicals, secreting trophic factors and secreting anti-inflammatory cytokines [103]. In GM1 gangliosidosis and Sandhoff disease mouse models, concentrations of the pro-inflammatory cytokines tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and anti-inflammatory transforming growth factor-β (TGF-β), were found to increase with disease progression and correlate with the increased expression of MHC-II, a marker of immune upregulation [104]. The brains of *Cln1/Ppt1* knock-out mice exhibit widespread astrogliosis with increasing age [65,67,105]. Studies of *post mortem* brain tissue from these mice and a CLN1 disease patient reported that PPT1-deficiency leads to more S100ß and receptors for advanced glycation end products (RAGE), in turn mediating the activation of nuclear factor-kappa B (NF-κB) and resulting in the production of IL-1β, IL-6, monocyte chemotactic protein-1 (MCP-1) and TNF-α pro-inflammatory cytokines [106], which most likely contribute to neuroinflammation in CLN1 disease. Preliminary findings in sheep suggest that the up-regulation of both pro- and anti-inflammatory cytokines occurs early in CLN6 disease and prior to overt neurodegeneration [107].

4.3. Adaptive immune responses

Aside from the documented activation of the innate immune system in different NCL models, adaptive immune responses may also contribute to inflammation in the central nervous system (CNS). Under normal conditions the blood–brain barrier (BBB) restricts the entry of plasma components, such as leukocytes, into the brain. During neuroinflammation and neurodegeneration the BBB can be compromised, resulting in lymphocyte

infiltration allowing immune cells to migrate into the brain. Subsequently these cells can become activated, releasing further inflammatory factors and creating a positive feedback loop which can result in more neuronal damage [108]. Size-selective breaches in the BBB have been reported in CLN3 disease, allowing the passage of IgG and serum proteins with pro-inflammatory properties into the brain [109], but there was very little evidence for lymphocyte infiltration of the CLN3 disease affected CNS until late in disease progression. It will be informative to document these adaptive responses in more detail in other forms of NCL. Recently it was reported that CD4 + T-helper 17 (T_H17) lymphocytes may mediate the BBB disruption and neuroinflammation in CLN1 disease [110].

4.4. Immunosuppression

A recent study showed that immunosuppression can alter disease severity in *Cln3* mutant mice [111], resulting in improvements in motor performance. This has led to a phase I safety trial of mycophenolate mofetil (CellCept) in human CLN3 disease cases. In a study of eight CLN3 disease patients treated with pulsed steroids for a year, an improvement was observed in motor symptoms in the oldest patient and cognitive benefits in two of the younger patients [112]. Another study in *Cln1* knockout mice, crossbred to lack T- and B-lymphocytes, indicated that although pathological measures were delayed and some functional improvements were seen, these mice ultimately reached the same disease end-point with only moderate extension of life-span [113]. Hence, although neuroinflammation is strongly implicated in disease pathology, therapies targeting this phenomenon alone may not be sufficient for disease suppression. Furthermore these interventions may need to be targeted precisely. The neuroinflammatory cascade is a complex network of feedback loops, and can be initiated at different points. In many cases it is not clear where anti-inflammatory drugs act in this cascade. Further investigations will be required to determine which parts of the cascade are activated early in pathogenesis and to select drugs targeted to those points for accurate pharmacological intervention.

5. Abnormal lipid metabolism and myelination

While overt demyelination is not apparent, there have been a number of reports of altered myelin structure, tissue lipid composition and lipid metabolism in different forms of NCL. The significance of these changes is unclear and recent studies have continued to search for molecular and mechanistic explanations for the role of lipids in NCL diseases.

The apparent lipid nature of the stored lipopigment drew early attention to the possible involvement of abnormal lipid metabolism in the pathogenesis of the NCLs. Early biochemical studies concentrated on the sphingolipids, *i.e.* cerebrosides and gangliosides, as these compounds were implicated in the other forms of amaurotic idiocy, but the work was abandoned as no useful information emerged. The most notable general finding is an increase in esterified cholesterol in the infantile and late infantile forms of the disease [114–117].

5.1. Abnormal phospholipid fatty acids

Specific changes in brain phospholipid fatty acids were also reported. The fatty acid profiles of the major brain phospholipids in advanced cases of classic infantile CLN1 disease were different from controls. In particular, there was an increase in 20:4(n − 6), a decrease in 22:4(n − 6) and a large decrease in 22:6(n − 3) and the name "polyunsaturated fatty acid lipidosis" was proposed to distinguish this disease from other forms of ceroid-lipofuscinosis in which these changes were not observed [118]. Subsequently pronounced alterations in brain ganglioside and neutral glycosphingolipid patterns in CLN1 disease were reported [119]. However these changes were rationalised as a consequence of the very large cellular and tissue changes associated with the brain atrophy and astrocytosis, all of which are particularly prominent in CLN1 disease. On the other hand only minor differences were observed in the fatty acid profiles of grey matter phospholipids from CLN6 affected sheep brains [120], and no differences in liver phospholipids or abnormalities in storage body associated phospholipids [14].

This issue has been revisited in recent analyses of the brain tissue derived from patients and mouse models of NCL. In CLN1 disease brain tissue, which had lost 65% of its phospholipids, the phosphatidylserine species were most dramatically affected [121]. All remaining phospholipid species contained an increased amount of long chain fatty acids, but decreased amounts of long chain polyunsaturated fatty acids. Again the lipid composition of human CLN3 disease brains was close to normal [121]. Alterations in the polyunsaturated fatty acyl chains were found in CLN8 disease human brain tissue along with severely reduced concentrations of ceramide, galactosyl- and lactosylceramide [122]. Studies in *Cln8/mnd* mutant mice revealed reduced concentrations of galactolipids, typical components of myelin [88]. A recent genome-wide association study indicated *CLN8* as a modifier of Gaucher disease, and suggested that CLN8 may function as a protective sphingolipid sensor and/or in glycosphingolipid trafficking [123].

5.2. Cholesterol metabolism

Altered cholesterol metabolism has been revisited in studies of *Cln1/Ppt1* and *Cln10/Ctsd* deficient mice. Global transcript profiling of *Cln1/Ppt1*$^{\Delta ex4}$ knock-out mouse neurons indicated that cholesterol metabolism might be deregulated with sterol biosynthesis being enhanced and steady-state amounts of sterols altered at the cellular level, but no changes in cholesterol concentrations were observed [124]. In addition, changes in the lipid composition of *Cln1/Ppt1*$^{\Delta ex4}$ knock-in mouse serum and dysregulated uptake of apolipoprotein A-I by *Cln1/Ppt1*$^{\Delta ex4}$ neurons were reported [125]. Increased amounts of unesterified cholesterol and cholesteryl esters may occur in *Cln10/Ctsd* knock-out mouse brains [125]. Abnormal expression of proteins that could be related to cholesterol transport was noted, with an increase of apolipoprotein E and a reduction of ATP-binding cassette transporter A1, and it was suggested that the trafficking of cholesterol is altered in *Cln10/Ctsd* knock-out brains.

Disease associated accumulation of bis(monoacylglycero)phosphate (lysobisphosphatidic acid, LBPA) was also reported in *Cln10/Ctsd* knock out mouse brains, but not in *Cln6/nclf* mouse brains, in contrast to the original report of accumulation associated with storage body isolates from CLN6 disease affected sheep [126,14]. In either case, accumulation is not necessarily indicative of a metabolic defect. Originally bis(monoacylglycero)phosphate was thought to be a marker of lysosomal membranes and has recently been identified as a component of late endosomal membranes. Thus increases of it are likely to be consequent to changes in the number of these vesicles, as a consequence of trafficking disruptions, and of the presence of late endosome–lysosome membranes in storage body isolations. Another study of gene expression profiling in cultured CLN6 disease fibroblasts also implicated disruptions of cholesterol dynamics, but although increased non-specific histological filipin staining was observed, no cholesterol accumulation could be found by physical high performance thin layer chromatography analysis (HPTLC) [127].

5.3. Myelination

In *Cln10/Ctsd* mice, the molecular changes associated with lipid metabolism were accompanied by complex morphological alterations of myelin structure, leading to the suggestion that myelination may be disturbed in these mice [128]. Recently, myelination defects have also been reported in *Cln8/mnd* mutant and *Cln5* knock-out mice [88,92]. Loss of Cln5 was associated with defective myelination *in vitro* and *in vivo*, and accompanied by early alterations in the serum lipid

composition and lipid transport in *Cln5* knockout mice [89]. Minor delays in myelination-associated changes in oligodendrocyte maturation were reported in *Cln8*/*mnd* mutant mice [88].

Taken together, brain lipids appear to be affected in most forms of NCL, albeit not in the same manner, but it would appear that this is as a consequence rather than a cause of the disease process. Dysregulation of the myelination process, suggested to occur in CLN10 disease, CLN5 disease and CLN8 disease, is consistent with pathological observations in patients and animal models of NCLs. However it is conceivable that altered lipid composition of membranes and altered lipid transport may have implications for membrane fusion events within and between cells and for lipid based cell signalling.

6. Abnormalities in intracellular metabolism and trafficking

There have been numerous indications of disturbances in the endosome–lysosome pathway and suggestions of problems with autophagy in the NCLs. Determination of the intracellular resident sites of the membrane bound CLN gene products (see 3.2 Functions of the gene products) places them as likely components of the endoplasmic reticulum–Golgi–endosome–lysosome (GERL) pathway, along with the other lysosomal storage diseases of non-lysosomal origin. A number of pathological investigations and cell biology experiments on isolated cells and in organisms of varying species reinforce this. There have also been suggestions that perturbations in lipids may be involved.

6.1. Autophagy

Autophagy is a strictly regulated cellular process involving the degradation of macromolecules and organelles *via* the lysosomal/vacuolar system and the involvement of autophagy in triggering neuronal death in the NCLs has been mooted, based on the observed abnormalities in autophagic pathways [129]. An increase in the number of autophagic vacuoles in the brains of *Cln10*/*Ctsd* knock-out mice was reported [130], along with the simultaneous accumulation of classical storage inclusions and autophagic vacuoles containing storage deposits. There was also a significant increase in the amount of autophagosome-associated LC3-II protein, and reduced colocalisation of LC3-II with the lysosomal associated membrane protein, LAMP1.

LC3-II was also increased in $Cln3^{\Delta ex7/8}$ knock-in mice, and isolated autophagic vacuoles from these mice had a less mature ultrastructural morphology than those from wild-type mice [131]. Co-localisation of LC3-positive vesicles with endocytic and lysosomal markers was reduced in cerebellar cell lines from these mice compared to controls, and the inhibition of autophagy led to cell death. The authors suggested that autophagic vacuolar maturation is disrupted in CLN3 disease and that activation of autophagy may be a pro-survival feedback response in the disease process. Further analysis of cerebellar cells from both the $Cln3^{\Delta ex7/8}$ knock-in mice and *Cln6*/*nclf* mice showed that the accumulation of the subunit c protein occurs within acidic organelles rather than in the mitochondria before transfer to lysosomes, consistent with a defect in the autophagosome-lysosomal pathway in these forms of NCL [132]. This could arise from distinct functions of NCL proteins in specific biological pathways essential for proper neuronal cell survival and also connected to the lysosome, the mitochondrion and subunit c turnover.

An age dependent increase in the amount of LC3-II in *Cln6*/*nclf* mice was accompanied by the formation of neuronal p62 positive aggregates, suggesting impaired fusion between autophagosomes and lysosomes. This became apparent between 20 weeks and 40 weeks, increasing again after a year [133]. The authors suggested that lysosomal dysfunction associated with CLN6 deficiency leads to an impairment of constitutive autophagy, which may promote neuronal degeneration *via* formation of the p62 positive aggregates that are toxic for cells.

Taken together, these data indicate that autophagy is induced in CLN3 disease, CLN6 disease and CLN10 disease, but whether the observed changes promote neuronal death or arise from a rescue attempt, remains an unanswered question. It also remains unclear whether the fusion between autophagosomes and lysosomes is impaired. Autophagy is rapidly induced in particular metabolic circumstances such as changes in nutritional status, and it is likely that these contribute to the observations above. It is also pertinent that hyperactive autophagy is not a feature noted in the large number of histological and ultrastructural studies of tissues from patients and animal models.

6.2. Endocytosis and intracellular trafficking

A defect in intracellular trafficking was studied in CLN3 disease fibroblasts, in which impaired receptor-mediated endocytosis was discovered, and Hook1, a protein involved in the regulation of endocytosis, was identified as a potential mediator of this defect [134]. Studies in a $Cln3^{\Delta ex7/8}$ cerebellar cell line also indicated disturbed endosomal/lysosomal membrane trafficking. Lysosome and endosome sizes and distributions were reported to be altered, and mitochondria elongated and functionally compromised. These organelle defects were apparent prior to subunit c accumulation, suggesting that storage does not result in membrane trafficking defects, but instead that trafficking defects are early events in the disease process [49]. CLN3 protein localization has been reported to partially overlap with lysosomes, synaptosomes and endosomes in neuronal cells [50,135,136], suggesting that the CLN3 protein resides in a number of vesicular compartments and may link multiple membrane trafficking pathways. These trafficking defects could particularly affect neuronal function through the importance of membrane vesicle transport in neurotransmission [49]. A study in *Schizosaccharomyces pombe* indicated that Btn1, the proposed yeast CLN3 ortholog, affected not only endocytosis, but also polarization of sterol-rich membrane domains and polarized cell growth [137].

A recent study in *Saccharomyces cerevisiae* suggested that Btn1 is involved in regulating the retrograde transport from the endosomal compartment to Golgi and in maintaining Golgi integrity [138]. The authors concluded that Btn1 controls retrograde sorting by regulating SNARE phosphorylation and assembly, possibly *via* modulating Yck3, a palmitoylated endosomal kinase, and suggested that these pathways may be adversely affected in CLN3 disease patients. These observations were recently complemented by suggestions that the anterograde transport of late endosomal/lysosomal compartments is affected by CLN3 deficiency [139]. Experiments were interpreted to suggest that the CLN3 protein interacts with motor components driving microtubular trafficking, including tubulin, dynactin, dynein and kinesin-2, as well as with active GTP-bound Rab7 and with the Rab7-interacting lysosomal protein (RILP) that anchors the dynein motor. The CLN5 protein was also implicated in endosomal trafficking recently, *via* its interaction with a lysosomal sorting receptor, sortilin, because the ablation of the CLN5 protein in HeLa cells altered the retrograde trafficking from endosomes to the Golgi [140].

Defective endocytosis has also been described in CLN1(PPT1) deficient fibroblasts [141]. Colocalisation of PPT1 with growth associated protein 43 and synaptophysin indicated specific targeting of PPT1 to axons in mature neurons, and perhaps a role for PPT1 in the exocytic pathway of neurons and in synaptic function. This observation in mammalian cells was supported by findings in a *Drosophila* model of CLN1 disease, with the identification of modifier genes which connected PPT1 function to synaptic vesicle cycling, endosome-lysosomal trafficking, synaptic development, and activity-dependent remodelling of the synapse [142]. Another screen in a loss-of-function *Drosophila* model of CLN1 disease identified modifier genes associated with cellular trafficking and endocytosis [143]. In order to verify these observations, the authors used Garland cells for assaying endocytosis in *Drosophila*, and concluded that the loss-of-function *Cln1*/*Ppt1* mutants exhibited a decrease in fluid-phase endocytosis without major defects in the

endosome-lysosomal compartment. Furthermore, ultrastructural analysis of the Garland cells revealed a reduction in uptake and suggested that the loss of *Cln1*/*Ppt1* impacts on an early step in vesicle formation during endocytosis.

It is evident from the studies discussed above that the involvement of the CLN proteins in intracellular trafficking and endocytosis is complex. Although there is considerable evidence for disruptions in these processes, particularly in CLN1 disease and CLN3 disease, the exact mechanisms and interactions are unclear. Multiple models of these diseases have suggested the involvement and interactions of many different proteins. It is also unclear whether trafficking disruptions are a cause or effect of the disease process, and the relevance of the various model systems used is not always obvious.

It is also likely that neurons would be especially sensitive to trafficking and endocytic deficits. Neurotransmission is a high energy process and is heavily dependent on membrane vesicle transport, hence disruption to these processes could have significant impacts on proper neuronal function and survival (see below). The previously mentioned regional susceptibility of certain neuronal populations to neurodegeneration suggests that any observed trafficking defects may have more severe consequences within certain neurons and brain regions. Thus caution is required when elucidating results from single cell models of the disease which do not accurately model the complexity of the disease pathogenesis.

6.3. Synaptic trafficking

Pathological alterations, including synaptic loss and the abnormal distribution of presynaptic proteins, occur in most forms of NCL. Mouse model studies have indicated that changes in the presynaptic proteins occur before indications of synaptic degeneration (see above). Synaptic vesicle trafficking is really a special case extension of the intracellular trafficking between the ER, Golgi and endosome-lysosomal compartments. An additional complication in neurons is the transport of signals from receptors at the extremes of the axons and dendrites to and from the nucleus, which also uses this machinery. Recent studies providing such possible links are discussed below.

Synaptic vesicles contain several palmitoylated proteins, which facilitate fusion of the vesicle with the synaptic membrane, exocytosis and recycling. These palmitoylated proteins are potential targets of PPT1. Electron microscopic studies revealed alterations in the size of the synaptic vesicle pool in neurons derived from *Cln1*/*Ppt1* knock-out mice, accompanied by a decrease in the frequency of miniature synaptic currents [144]. Another study showed that PPT1 deficiency led to persistent membrane association of the palmitoylated synaptic vesicle proteins, including synaptotagmin, synaptosomal-associated protein 25 (SNAP-25), and Syntaxin 1, and the authors hypothesized that this caused the progressive decline in the total and readily releasable synaptic vesicle pool in *Cln1*/*Ppt1* knockout mice [145]. Similar observations were made in *Cln6*/*nclf* mice, suggesting a reorganisation of the presynaptic compartment before synaptic loss occurs [146]. Modifier gene screening in a *Drosophila* model of CLN1disease led to an observation that both synaptotagmin and stoned A, which facilitate synaptotagmin recycling, suppressed the CLN1 disease phenotype [142]. Together with the identification of other modifiers, the authors concluded that PPT1 functions in both the regulation of synaptic vesicle endocytosis and the regulation of endocytosis.

Electron microscopic analysis reported a marked and progressive increase in the number of synaptic vesicles (particularly docked vesicles) per bouton in brains of *Cln10*/*Ctsd* knock-out mice [147], accompanied by a decrease in the frequency of miniature synaptic currents, suggesting impaired release of synaptic vesicles. However, no defect in the spontaneous release of synaptic vesicles was observed in electrophysiological measurements. In addition, cathepsin D has been indirectly implicated in synaptic vesicle trafficking *via* the regulation of α-synuclein [148,149], thought to promote SNARE-complex assembly at presynaptic terminals.

A recent study on CLN4 (CSPα) showed that the CSPα chaperone complex binds SNAP-25 and dynamin 1, which are necessary for synaptic vesicle fusion and fission respectively [150]. CSPα regulates synaptic vesicle number and participates in synaptic vesicle endocytosis in hippocampal cultures, leading the authors to suggest that it may facilitate exo- and endocytic coupling.

In summary, further to studies indicating that intracellular trafficking and endocytosis are affected in CLN1 disease and CLN3 disease there is increasing evidence of an early involvement of synaptic failure and possibly defects in the recycling of synaptic vesicles as part of pathogenesis in CLN1 disease, CLN4 disease and CLN10 disease. The reports discussed above suggest that vesicular fusion events controlled by SNARE proteins may be involved. In addition endosomal trafficking is likely to affect neural development and influence the polarity and migration of neurons as well as axon outgrowth and guidance, and is strongly linked with synaptosomal vesicle recycling [151].

7. Pathways leading to apoptosis

Programmed cell death, or apoptosis, may originate from either extracellular or intracellular stimuli. Extracellular triggers include hormones, growth factors, nitric oxide and cytokines, while intracellular initiation may be a response to stress, caused for example by hypoxia, increased intracellular calcium concentration, ER stress or membrane damage. Independent of the apoptotic stimulus, cytochrome c release and caspase activation can each play a role in the execution of apoptosis. However the genetic modulation of the central apoptotic pathways involving p53 or Bcl-2 failed to alter the disease course in a mouse model of CLN2 disease, indicating that either neuronal death does not occur *via* apoptosis in this disease, or if it does, it occurs *via* pathways not involving p53 or Bcl-2 [152].

7.1. The TNF-α pathway

The cytokine, tumour necrosis factor α (TNFα), can initiate ceramide and sphingosine-mediated apoptosis. It was noted that fibroblasts derived from patients with CLN1 disease or CLN2 disease were partially resistant to TNFα-induced apoptosis, while CLN3 or CLN5 deficiency did not protect fibroblasts from TNFα-induced apoptosis [153,154]. Such protective effects were not observed in *CLN10*/*CTSD* deficient fibroblasts [155]. Studies of *Bax*/*cathepsin D* double knock-out mice showed that the suppression of the caspase dependent apoptosis by inactivation of Bax prevented apoptosis, but not neuronal death, indicating that a caspase dependent route is not responsible for neuronal death in *Cln10*/*Ctsd* knock-out mice [156].

7.2. Phosphatidylinositol signalling

Phosphatidylinositol-3 kinase (PI3-K) regulates multiple cell signalling events in many pathways, including the apoptotic pathway activated by survival factors, such as growth factors and cytokines. Disturbances of the phosphatidylinositol signalling pathway have been reported in CLN10 and CLN3. A marked decrease in phosphoinositol PI-3-K signalling was suggested to relate to autophagic stress in *Cln10*/*Ctsd* knock-out mice [157]. Inositol monophosphatase inhibitors partially rescued the autophagic process and reduced the neuronal vulnerability in $Cln3^{\Delta ex7/8}$ mouse cerebellar cells, in which autophagy was impaired [131,158].

8. Other postulates

8.1. Oxidative pathways

Mitochondrial dysfunction and oxidative stress have been proposed to be involved in the pathogenesis of NCLs. Elevated concentrations of

reactive nitrogen species have been reported in *Cln3*$^{\Delta ex7/8}$ knock-in mouse tissues [159]. Mitochondrial abnormalities and a decreased survival after oxidative stress were reported in cerebellar precursor cells carrying the *Cln3*$^{\Delta ex7/8}$ mutation [49]. Elevated amounts of reactive oxygen species or of superoxide dismutases have also been reported in CLN1 and CLN8 diseases [160,161].

8.2. ER stress response

An endoplasmic reticulum (ER) stress response is thought to be triggered by an imbalance of intracellular homeostasis, and a contribution to NCL pathogenesis has been suggested. Unfolded protein response (UPR) markers were observed to be upregulated in *Cln1*/*Ppt1* knock-out mouse brains [162]. Several early ER-stress indicators were upregulated in *Cln8*/*mnd* mutant mice [163]. Mutations in the newly discovered *CLN12*/*ATP13A2*/*PARK9* gene have also been linked with ER-stress-induced cell death [164].

8.3. Excitotoxicity

As mentioned in Sections 3.3 and 4.1 excitotoxicity has been postulated to be involved in the pathology of the NCLs and the biochemical evidence is discussed there. Although there is no direct evidence for any imbalance or metabolic disturbance that could lead to the onset of neurodegeneration, a vulnerability of *Cln3*$^{\Delta ex1-6}$ cerebellar granule neurons to glutamate has been reported [165]. This has led to the use of different classes of glutamate receptor antagonists as potential therapeutic agents in mouse models of classic juvenile CLN3 disease, and small influences of either NMDA or AMPA receptor antagonists on rotarod performance and brain inflammation have been reported [166–168]. A similar involvement of glutamate receptors has been proposed in CLN1 disease [166,169], but their antagonists are yet to be tested *in vivo* in this form of NCL. In summary, multiple signalling pathways appear affected in NCL diseases. Many of these pathways overlap and influence each other. It appears intriguing, however, that some defects (CLN1 and CLN2) make cells more resistant to cell death stimuli than control cells are [153,154].

9. The physiological dimension

Although biochemical and cell biology experiments have yielded much information about neuropathogenesis in the NCLs, it is now clear that there is a physiological dimension. This is apparent from the regionality of neuroinflammation and the connections with neurodegeneration and the development of symptoms observed in the CLN6 sheep model [81,82,85,87] and in a number of mouse studies [76]. As stated earlier, the location and connectivity of neurons are better determinants of disease development than their phenotypic identity. This is highlighted by the very specific loss of GnRH secreting cells from the hypothalamus of CLN6 affected sheep without neuroinflammation or any sign of dysfunction of any other cells [85]. In a way it is remarkable how much of the CNS remains functional despite the obvious burden of storage bodies across all forms of the disease. Whatever the intracellular lesions may be, most cells, including most neurons, tolerate them reasonably well. The importance of circulating factors including those secreted by other non-neuronal cells, like glia, is not defined. These considerations also indicate that there are major aspects of the diseases not accessible from single cell studies.

10. Conclusion

At the biochemical level the NCLs are classified as a group of lysosomal proteinoses in which specific proteins accumulate in lysosome derived storage bodies, with characteristic ultrastructural and fluorescent properties. Storage is not confined to neurons in the classic forms but is generalised, with storage bodies accumulating in most tissues throughout the body. Specific accumulation of the sphingolipid activator proteins, SAP A and SAP D, results from mutations in *CLN1* and *CLN10* while subunit c of mitochondrial ATP synthase accumulates in disease resulting from mutations in *CLN2*, *CLN3*, *CLN5*, *CLN6*, *CLN7* and *CLN8*. This specificity and generality of subunit c storage differentiate this group from other lysosomal storage diseases where some storage of subunit c has also been found, but only in subsets of neurons.

The composition of the stored material and the generality of storage are not so clear in the more recently classified forms, but there is evidence of specific protein storage in some, and further work is required to determine how these diseases fit in a grouping of lysosomal proteinases. It also follows that subunit c accumulation reflects a series of breakdowns in the catabolic pathway for this molecule, but despite several attempts to study this pathway little is known of it. Presumably the SAP storing forms are a related subgroup.

What is also apparent is that storage body accumulation is not the cause of the neurodegeneration characteristic of the NCLs, and that this arises from some other manifestation of the mutations. The turnover of subunit c requires the removal of this highly hydrophobic molecule from the inner mitochondrial membrane, either as part of the oligomeric ATP complex or after disassembly from it, and transport to the lysosome. Autophagy and intracellular vesicular trafficking are central to this process. There is accumulating independent evidence that aberrant vesicular trafficking plays a role in NCL pathogenesis with defects along the endocytosis pathway, and in the vesicular trafficking from Golgi to endosomes and back having been reported, but nearly everything possible has been invoked in one study or another and many studies contradict each other. Trying to tie all these reports together into coherent whole would be a major review in itself, certainly beyond the scope of this article. Most reports concentrate on one aspect exclusively, with little information of up- or down-stream modulators, or of parallel pathways.

However taken together these studies allow little doubt that intracellular trafficking and signalling abnormalities are important in the NCLs. In neurons the intracellular transport system is interwoven with transporting signalling molecules into the cell and to and from the nucleus. This complexity may also be reflected in the recycling of synaptic vesicles where the size of the readily releasable synaptic vesicle pool is pathologically altered, as seen in the CLN1 and CLN10 disease models. Possibly, the SNARE protein assembly may be affected by NCL proteins indirectly; for example CLN10-(CTSD) and ATP13A2 may affect SNARE assembly in synapses *via* α-synuclein.

Alterations in synaptic communication would affect neuronal connectivity within defined circuits, as indicated by the histopathological studies showing the degeneration of the somatosensory thalamocortical system in many mouse models of NCL and regional cortical degeneration in sheep. The exact neuronal circuitry and where the disease begins, may vary between the different forms of NCL, but the basic principle could be applicable across all disease forms and species.

The strong evidence that glial activation precedes neurodegeneration in the NCLs, implicates interactions between neurons and glia. Studying these interactions will be important for the future. There are also strong indications of a physiological dimension, perhaps through neural circuitry or the influence of circulating trophic factors. Although there is considerable evidence for defects in individual metabolic processes (signalling, autophagy), it has become apparent that no single level of investigation will resolve the pathogenesis of NCLs, but when the evidence from multiple disciplines is viewed collectively, we may understand which cellular processes are affected and will begin to understand the consequences at a physiological and functional level.

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