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Review

Defective cholesterol trafficking in Niemann-Pick C-deficient cells

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ABSTRACT

Pathways of intracellular cholesterol trafficking are poorly understood at the molecular level. Mutations in Niemann-Pick C (NPC) proteins, NPC1 and NPC2, however, have led to insights into the mechanism by which endocytosed cholesterol is exported from late endosomes/lysosomes (LE/L). Mutations in NPC1, a multi-spanning membrane protein of LE/L, or mutations in NPC2, a soluble luminal protein of LE/L, cause the neurodegenerative disorder NPC disease. This review focuses on data supporting a model in which movement of cholesterol out of LE/L is mediated by the sequential action of the two NPC proteins. We also discuss potential therapies for NPC disease, including evidence that treatment of NPC-deficient mice with the cholesterol-binding compound, cyclodextrin, markedly attenuates neurodegeneration, and increases life-span, of NPC1-deficient mice.
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1. Introduction

Niemann-Pick type C (NPC) disease is a fatal, autosomal recessive disorder that affects approximately 1 in 150,000 live births [1]. The majority of cases result from mutations in the *NPC1* gene (95%) while the remainder (5%) are caused by mutations in the *NPC2* gene. Loss of function of either of these proteins results in an accumulation of cholesterol and other lipids, including sphingomyelin, sphingosine and gangliosides (GM2 and GM3), within the late endosomes/lysosomes (LE/L). In mice and humans, this impairment leads to progressive neurodegeneration, hepatosplenomegaly and, ultimately, premature death. Typically, clinical manifestations become evident in early childhood, although age of onset can range from the perinatal period to adulthood. Disease progression is often more rapid when onset of symptoms occurs in early life [2].

Cells acquire cholesterol through endogenous synthesis, as well as through the uptake of exogenous sources of cholesterol, particularly low density lipoproteins (LDLs). LDLs are brought into the cell via receptor-mediated endocytosis and are delivered to the LE/L, where cholesterol esters (CE) within the core of the LDL particle are hydrolyzed by acid lipase [3]. Unesterified cholesterol exits the LE/L, apparently through an NPC1/NPC2-dependent mechanism, and is distributed to the plasma membrane as well as the endoplasmic reticulum (ER). The ER serves as a cholesterol sensor, allowing the cell to regulate cholesterol synthesis and uptake via the sterol regulatory element-binding protein pathway [4]. Additionally, cholesterol at the ER can be re-esterified by acyl-CoA:cholesterol acyltransferase (ACAT) [3].

Initial studies by Pentchev et al. found that fibroblasts from NPC1-deficient patients have a defect in cholesterol esterification despite having normal ACAT activity [5]. Staining of these cells with filipin, which labels unesterified cholesterol, suggested that although LDL-cholesterol is internalized and hydrolyzed normally in these fibroblasts, its movement to the ER is blunted [5]. Consequently, LDL is unable to decrease cholesterol biosynthesis and LDL-receptor activity, both of which are regulated at the ER [6]. Cytochemical techniques confirmed that there is a massive storage of cholesterol within the lysosomes of NPC-deficient fibroblasts [7]. More recent studies have begun to unravel the molecular and cell biological basis of these findings.

2. NPC1 and NPC2 proteins

Positional cloning techniques have identified the human *NPC1* gene (on chromosome 18q11) and its orthologs in mice, the yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans* [8,9]

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; ALLO, allopregnanolone; CD, cyclodextrin; CE, cholesterol esters; CNS, central nervous system; ER, endoplasmic reticulum; GSL, glycosphingolipid; LDL, low density lipoprotein; LE/L, late endosomes/lysosomes; NPC, Niemann-Pick type C

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and *Drosophila* [10]. Expression of NPC1 in cultured fibroblasts from patients with NPC disease abolished the lysosomal accumulation of cholesterol [8]. NPC1 is an integral membrane protein that is primarily localized to the LE/L, although this protein also appears to cycle through the *trans*-Golgi network [11,12]. Human NPC1 consists of 1278 amino acids and contains 13 putative transmembrane domains that are separated by three luminal glycosylated loops [8,13]. Moreover, NPC1 contains a sterol-sensing domain that has a similar sequence to that in 3-hydroxy-3-methylglutaryl-CoA reductase, sterol regulatory element-binding protein cleavage activating protein and Patched [8].

Through studies that were designed to characterize the lysosomal proteome, the gene responsible for NPC2 disease was subsequently identified [14]. NPC2 (previously called HE1) is a ubiquitously expressed 151-amino acid, soluble lysosomal protein [14]. Originally, NPC2 was identified as a cholesterol-binding protein that is a major secretory component of epididymal fluid [15]. Fluorescence quenching assays showed that NPC2 can transfer cholesterol between phospholipid vesicles. In addition, the transfer of cholesterol from phospholipid vesicles to NPC2 is dramatically increased by the presence of bis(monoacylglycero)phosphate (a LE/L phospholipid that accumulates in NPC-deficient cells) in donor vesicles [16].

Homozygous mutations in either NPC1 or NPC2 result in virtually identical cellular and clinical phenotypes [17]. Likewise, when NPC1-deficient mice are crossed with *Npc2* hypomorphic mice, which retain 0–4% residual NPC2 protein, the phenotype is indistinguishable from that of either NPC1- or NPC2-deficient mice [18], suggesting that these proteins function in tandem, or sequentially, in the same pathway. Binding studies and crystal structures of NPC1 and NPC2 have provided valuable insights into the functions of these proteins. NPC2 binds cholesterol with high affinity in a process that is sensitive to modification of the hydrophobic side-chain of cholesterol [15,19]. A crystal structure of bovine NPC2 bound to cholesterol sulfate shows that cholesterol binds in a deep hydrophobic pocket, with only the sulfate (substituted for the 3β-OH moiety) of the sterol exposed to solvent [20]. NPC1 also binds cholesterol and fluorescent sterol analogs, as well as oxysterols [19,21,22]. More recently, Kwon et al. reported the crystal structure of the N-terminal loop of NPC1 and revealed that this loop binds to cholesterol so that the 3β-OH group is buried within the binding pocket and the iso-octyl side-chain is exposed on the surface [23]. Since NPC1 and NPC2 appear to bind cholesterol in opposite orientations, a model for the functions of the two NPC proteins was proposed. In this model, unesterified cholesterol in the interior of the LE/L is initially bound by NPC2 which subsequently transfers the cholesterol to the N-terminal cholesterol-binding domain of NPC1 in the limiting membrane of the LE/L (Fig. 1) [23]. This model predicts that NPC1 inserts the iso-octyl side-chain of cholesterol into the lysosomal outer membrane, although this feature remains to be confirmed [23]. In support of this model, NPC2 greatly accelerates the bi-directional transfer of cholesterol from NPC1 to liposome acceptors, particularly those containing the LE/L-specific phospholipid, bis(monoacylglycero)phosphate [24]. The role that the sterol-sensing domain of NPC1 plays in this process remains unclear since mutations in this domain prevent the binding of a photoactivatable analog of cholesterol to NPC1 in intact cells [25]. However, direct binding of this cholesterol analog to the sterol-sensing domain was not demonstrated [25], leaving open the possibility that this domain modulates cholesterol binding to the N-terminal loop of NPC1. An alternative model that has been proposed, and not yet ruled out, for the sequential action of NPC1 and NPC2 is that NPC1 transfers cholesterol to NPC2. No direct interaction between the NPC1 and NPC2 proteins has been detected.

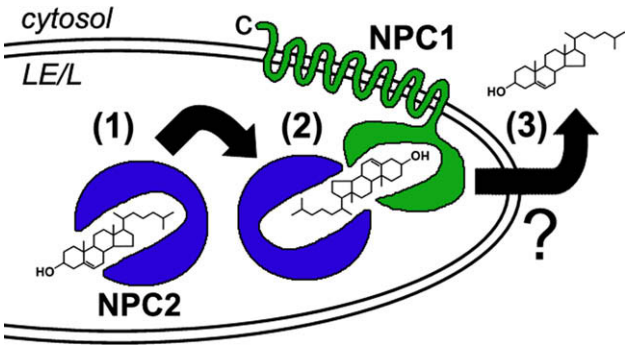

Fig. 1. Potential mechanism for NPC1/NPC2-mediated cholesterol export from LE/L. (1) NPC2 (soluble protein) binds unesterified cholesterol in the LE/L with the iso-octyl chain in the binding pocket, an event that may be enhanced by bis(monoacylglycero)phosphate. (2) NPC2 transfers cholesterol to the N-terminal loop of NPC1 (a multi-pass membrane protein) which binds cholesterol with the hydroxyl group in the binding pocket. (3) Cholesterol is exported from LE/L by an unknown mechanism.

Other experiments, in which NPC1 was expressed in *Escherichia coli*, indicated that NPC1 might be a fatty acid transporter [26]. In these studies, NPC1 was able to transport oleic acid, but not cholesterol or CE, across the *E. coli* membrane [26]. Nevertheless, subsequent work showed that the flux of fatty acids through the NPC1-deficient LE/L is normal, suggesting that fatty acid export from the LE/L does not require NPC1 in mammalian cells [27]. The finding that NPC1 that is expressed in *E. coli* cannot mediate the transbilayer movement of cholesterol might indicate that NPC1 requires the presence of functional NPC2 in order to transport cholesterol.

3. Abnormal lipid trafficking in NPC-deficient cells

The mechanisms responsible for the various pathways of inter-organelle trafficking of cholesterol within cells are largely undefined. The endocytic uptake of LDLs, and the subsequent hydrolysis of CE, are normal in NPC1-deficient cells [5]. However, the export of cholesterol and other lipids from the LE/L is defective. Consequently, normal amounts of cholesterol fail to reach the plasma membrane and the ER for regulation of cholesterol homeostasis [5,6]. Since cholesterol trafficking to the ER is impaired in NPC-deficient cells, the cells sense a lack of cholesterol. Thus, cholesterol synthesis and LDL-receptor expression are increased despite abnormally elevated cholesterol levels in the LE/L [6]. The mechanism by which cholesterol reaches the ER from the LE/L remains to be defined. Recent findings suggest that cholesterol is first trafficked from the LE/L to the *trans*-Golgi network prior to reaching the ER in a process that is dependent upon a SNARE complex, although other possibilities exist [28]. Endogenously-synthesized cholesterol can also accumulate within the LE/L, albeit much more slowly than does LDL-derived cholesterol [29]. Unlike LDL-derived cholesterol, the transport of endogenously-synthesized cholesterol to the plasma membrane is not impaired in NPC1-deficient fibroblasts [29,30]. Presumably through formation of endocytic vesicles and membrane recycling, this source of cholesterol can, however, also become trapped in the LE/L. Furthermore, cholesterol that is taken up through bulk-phase endocytosis also enters the LE/L and, as a consequence, becomes trapped within the LE/L compartment of NPC1-deficient cells [31]. Interestingly, the intracellular transport of cholesterol derived from high density lipoproteins appears to be unaffected by NPC1 deficiency, suggesting that cholesterol derived from these lipoproteins is processed in the cell through a pathway different from that used by LDL-cholesterol [32].

In addition to cholesterol, a variety of other lipids, including sphingomyelin, sphingosine, bis(monoacylglycero)phosphate, and

complex glycosphingolipids (GSLs, particularly the gangliosides GM2 and GM3) accumulate in the LE/L of NPC-deficient cells (reviewed in [33]). There has been, and still is, debate over which lipid is the first to accumulate in NPC-deficient cells, and which lipids are responsible for the cellular dysfunction underlying NPC disease. Although the majority of data implicate cholesterol accumulation as the primary defect, there is some evidence that points to other lipids. For example, in a drug (U18666A)-induced model of NPC disease the accumulation of sphingosine in the LE/L apparently preceded both calcium depletion of these organelles and cholesterol accumulation [34]. Moreover, calcium stores in the acidic compartment of NPC1-deficient cells were reduced, and the chelation of calcium in the lumen of the endocytic pathway of wild-type cells caused an NPC disease-like phenotype [34]. It should be noted, however, that although U18666A induces cholesterol accumulation in the LE/L, this compound does not precisely mimic NPC deficiency and has additional effects on cholesterol homeostasis, including inhibition of cholesterol synthesis [35,36]. It would be interesting to determine if sphingosine accumulation is also the initial event that causes lipid accumulation in the LE/L of NPC1-deficient cells.

Several studies indicate that GSL accumulation in the LE/L is not the causative event in NPC disease. For example, NPC1-deficient mice that also have disrupted *Galgt1* and *Siat9* genes, and are unable to synthesize GM2/GD2 or GM3 complex gangliosides, accumulate the same amount of cholesterol in LE/L in the brain and other tissues as do NPC1-deficient mice [37,38]. Moreover, rather than extending the life of NPC1-deficient mice, deletion of these genes shortened the life-span, most likely because GSLs are important for central nervous system (CNS) health and function [37,38]. Similarly, when mice lacking *N*-acetylgalactosamine transferase (unable to synthesize GM2 and complex gangliosides) were crossed with NPC1-deficient mice, the absence of GM2 did not increase the life-span of the NPC1-deficient mice [39]. Thus, elimination of the GSL storage did not prevent development of the NPC disease phenotype. On the other hand, in another study, elimination of the GSL biosynthetic enzyme *N*-acetylgalactosamine transferase in NPC1-deficient mice reduced filipin staining of cholesterol in some, but not all, types of neurons in the brain, although cholesterol content was not measured directly [40].

The idea that cholesterol is the initial lipid that accumulates in NPC deficiency is strongly supported by the observation that both NPC1 and NPC2 bind cholesterol [20,23], while this evidence is lacking for other lipids such as GSLs. Currently, the majority of experimental evidence supports a model in which mutation of either NPC1 or NPC2 initially leads to cholesterol accumulation in the LE/L, which in turn causes sequestration of a variety of other lipids. Cholesterol accumulation in the LE/L also causes mis-trafficking of GSLs [41] and GSL accumulation can cause cholesterol entrapment in the LE/L [42]. Cholesterol storage in the LE/L is increased in several sphingolipid storage disorders, and depletion of cholesterol from sphingolipid storage disease cells restored the normal trafficking of a fluorescent GSL analog (BODIPY-lactosylceramide) from the LE/L to the Golgi apparatus [41]. Moreover, when wild-type cells were overloaded with cholesterol, the trafficking of BODIPY-lactosylceramide to the LE/L increased [41]. The affinity of cholesterol for GSLs is indicated by their association in detergent-resistant domains of cell membranes. Thus, in NPC disease, not only is it possible that the initial sequestration of cholesterol leads to the accumulation of GSLs, but these GSLs might subsequently induce the accumulation of even more cholesterol. We speculate that this cyclic buildup of lipids within the LE/L might explain why treatments that focus on reduction of GSL storage can provide a therapeutic benefit in NPC1-deficient models [43]. Devlin et al. provide compelling evidence in support of this theory, by demonstrating that although impaired cholesterol trafficking is most likely the primary defect in NPC1-deficient cells, correction of a secondary defect can substantially improve lipid and protein trafficking [44].

Another lipid that accumulates in the LE/L of NPC1-deficient cells is sphingomyelin. In response to the abnormal cholesterol accumulation in membranes of the LE/L in NPC1-deficient fibroblasts, the activity of acid sphingomyelinase was reduced although the amount of this enzyme was normal [44]. When normal levels of acid sphingomyelinase activity were restored in the NPC1-deficient fibroblasts, either through cDNA transfection or enzyme replacement, a dose-dependent reduction in lysosomal cholesterol content occurred and the abnormal recycling of the transferrin receptor was partially corrected [44]. Even when normal sphingomyelinase activity was restored, the intensity of filipin staining remained 25–30% higher than normal, most likely due to the primary cholesterol trafficking defect [44]. The same logic might explain the partial success of miglustat treatment of children with NPC disease [45,46]. Miglustat is a small iminosugar that reversibly inhibits the first step of GSL synthesis (glucosylceramide synthase), improves the clinical symptoms, and increases the life-span of NPC1-deficient mice and cats [43]. This "substrate reduction therapy" is a potentially useful treatment for NPC disease since the residual activity of glucosylceramide synthase would likely produce sufficient GSLs for functioning of the CNS.

There is evidence that cholesterol accumulation in the LE/L can impair vesicular trafficking pathways. Vesicles of the endocytic pathway are transported along the cytoskeleton, a process that is regulated by Rab GTPases (reviewed in [47]). Over-expression of Rab7, which is involved in vesicle trafficking from the early endosomes to LE, or over-expression of Rab9, which is involved in LE to Golgi trafficking, corrected the lipid trafficking defects in NPC1-deficient fibroblasts [48]. Likewise, over-expression of Rab4 (which is involved in endocytic recycling to the plasma membrane) in NPC1-deficient cells also decreased cholesterol accumulation, and restored normal trafficking of BODIPY-lactosylceramide to the Golgi apparatus [49]. Although Rab4 protein levels are elevated in NPC1-deficient cells, the sequestration of cholesterol in the LE/L prevents the extraction of Rab4 from the endosomal membrane, a key step required for Rab4 function [49]. As a consequence, the Rab4-mediated recycling of lipids and proteins is impaired [49]. Modulation of pathways that involve Rab4, Rab7 or Rab9 was suggested as a method of overcoming the trafficking defects in NPC-deficient cells. Along these lines, over-expression of Rab9 in NPC1-deficient mice increased life-span and reduced ganglioside storage in the brain [50]. Over-expression of Arf6 (which is involved in regulation of early endosomal membrane internalization and recycling) also reduced lipid accumulation in NPC1-deficient cells [51]. Furthermore, Goldman and Krise have demonstrated that fusion/fission events between the LE and lysosomes are severely impaired in NPC1- and NPC2-deficient cells [52]. Intriguingly, it appears that NPC1 and NPC2 might be involved in different aspects of this process, so that NPC1 is important for the retrograde fusion of LE and lysosomes, while NPC2 plays a role in fission events that might be important for re-formation of lysosomes [52]. Retrograde fusion of lysosomes with LE is thought to be important for egress of membrane-impermeable cargo out of the cell, and impairment of this pathway might cause a build-up of toxic metabolites in the LE/L [52]. Indeed, the release of molecules such as dextran and sucrose from NPC1-deficient cells is impaired [12,52,53]. Thus, lipid accumulation within the LE/L can severely reduce the trafficking of lipids and other molecules through the endocytic pathway.

Mitochondria are crucial for providing cells with energy in the form of ATP, and also play a central role in apoptosis. Due to these important functions, mitochondrial dysfunction has been implicated in cell death in a variety of neurodegenerative disorders.

Cholesterol trafficking from the LE/L to mitochondria does not appear to be impaired by NPC1 deficiency [54]. Indeed, the cholesterol content of mitochondria in brains, neurons and hepatocytes from NPC1-deficient mice is increased [54–57], probably due to the increased amounts of unesterified cholesterol in the LE/L that are available for import into mitochondria. Interestingly, RNA silencing of the endosomal protein MLN64 decreased the cholesterol content of mitochondria, both in the absence and presence of NPC1 indicating that MLN64 can mediate the transport of endosomal cholesterol into mitochondria independent of NPC1 [54]. Since cholesterol regulates membrane fluidity, these changes in mitochondrial cholesterol content might have a direct impact on mitochondrial function and stability, as suggested in several studies. For example, mitochondrial membrane potential, the activity of ATP synthase, and levels of ATP are all decreased in NPC1-deficient mouse brains and neurons [57]. Mitochondria from NPC1-deficient cells also have decreased levels of glutathione, which serves as an important anti-oxidant defense. Consequently, these cells have greater susceptibility to killing by tumor necrosis factor-α, as well as by oxidative stress induced by amyloid-β1-42 [55,56].

4. Consequences of NPC deficiency in the liver and the brain

In individuals with NPC disease, cholesterol accumulates in almost all tissues of the body in amounts that are proportional to the rate at which each tissue obtains cholesterol through the LDL-receptor pathway [58]. The liver is responsible for the uptake of ~80% of LDL-cholesterol [58]. Although the rate of LDL-cholesterol uptake by the liver is similar in wild-type and NPC1-deficient mice, the large flux of cholesterol through the liver causes a massive accumulation of cholesterol within the LE/L of NPC1-deficient mice [58]. Consequently, markers of liver damage (alanine aminotransferase and aspartate aminotransferase activities) are substantially increased in NPC1-deficient mice [59]. Moreover, ~50% of infants with NPC disease exhibit hepatomegaly which can lead to liver failure and death [60]. Underscoring the unique role that the liver plays in lipoprotein metabolism, the extent of cholesterol accumulation in NPC1-deficient mouse hepatocytes is much greater than in NPC1-deficient fibroblasts [61]; cholesterol levels in hepatocytes from *Npc1*$^{-/-}$ mice are 5- to 10-fold higher than in *Npc1*$^{+/+}$ mice [61]. Enigmatically, whereas in NPC1-deficient fibroblasts cholesterol esterification is markedly attenuated, cholesterol esterification in NPC1-deficient hepatocytes is increased [61]. In addition, NPC1 deficiency in hepatocytes increases the expression of ATP-binding cassette transporter A1 and the formation of high density lipoproteins [62]. It is likely that the massive overload of cholesterol in the LE/L of hepatocytes causes a leakage of excess cholesterol from the LE/L so that cholesterol can be transported to the ER where cholesterol homeostasis is regulated.

Defects in cholesterol metabolism in the brain have increasingly been linked to neurodegenerative disorders such as Alzheimer disease and Huntington disease. Cholesterol metabolism in the brain is unique, particularly since plasma lipoproteins are unable to cross the blood–brain barrier. Consequently, essentially all of the cholesterol in the brain must be synthesized endogenously [63]. The brain contains ~25% of the body's cholesterol, but comprises only ~5% of the body mass. The majority (70–80%) of cholesterol in the brain is present in myelin; the rate of cholesterol synthesis in the brain is highest during active myelination, and declines once myelination is complete [64]. In the adult brain, cholesterol synthesis is thought to occur primarily in astrocytes [65], which can supply cholesterol to neurons in the form of apolipoprotein E-containing lipoproteins (reviewed in [66]). Excess cholesterol in the brain is excreted in the form of 24-hydroxycholesterol which is able to cross the blood–brain barrier and enter the circulation [67]. Surprisingly, although cholesterol accumulates in tissues throughout the body in NPC disease, the amount of cholesterol in the brain does not increase, but even *decreases*, with age [58]. Several factors may explain this apparent discrepancy. First, extensive demyelination occurs in the NPC1-deficient brain, and since myelin is particularly rich in cholesterol, the demyelination would likely mask any increase in cholesterol content of other cells in the brain [68]. Second, the rate of cholesterol excretion from the brain is increased in NPC1-deficient mice [68]. However, as in other NPC1-deficient cells, cholesterol does accumulate in the LE/L of neurons of newborn NPC1-deficient mice [69] and in the brains of young NPC1-deficient mice, prior to extensive demyelination [68]. In addition, increased cholesterol staining was observed in NPC1-deficient brain slices compared to wild-type brains [70]. However, these studies did not determine if other lipids also accumulated in brains of young NPC1-deficient mice.

Since the blood–brain barrier isolates the brain from cholesterol in the circulation, treatments that modulate cholesterol homeostasis in other tissues will not necessarily translate into improvement of neurological symptoms of diseases that involve defects in cholesterol metabolism. Thus, when NPC1-deficient mice were crossed with LDL-receptor knockout mice the neuropathological features of NPC disease did not improve [71]. The lack of a beneficial effect of cholesterol-lowering treatments in the brain might be due to contributions of other lipoprotein receptors in the brain, or to the accumulation of endogenously-synthesized cholesterol within neurons. In support of the latter explanation, NPC1-deficient mouse sympathetic neurons [69], embryonic hippocampal neurons [33], and cerebellar granule neurons (K.B. Peake and J.E. Vance, unpublished observations) showed the typical punctate filipin staining pattern of cholesterol sequestration in the LE/L, even in neurons that had been isolated from newborn mice or cultured for 20 days in the complete absence of lipoproteins. Pharmacological attempts at lowering plasma cholesterol also failed to improve the NPC phenotype [72], possibly because some cholesterol-lowering drugs cannot cross the blood–brain barrier. On the other hand, when cholesterol synthesis was blocked using a squalene synthase inhibitor in young NPC1-deficient mice prior to formation of the blood–brain barrier, cholesterol accumulation in neurons was attenuated, as was astrocyte activation [73]. Importantly, however, blocking cholesterol synthesis at this early stage of mouse development might have disrupted the myelination process, which could negate any beneficial effects of this treatment for NPC1 deficiency [73]. When cholesterol homeostasis was altered using a liver X receptor agonist, however, the life-span of NPC1-deficient mice was extended and the neurodegeneration was delayed [74]. While the liver X receptor agonist did not reduce cholesterol concentration or synthesis in the brain, excretion of cholesterol from the CNS was increased [74]. Thus, modulation of cholesterol metabolism in NPC-deficient brains might reduce the severity and progression of NPC disease. Nevertheless, the importance of adequate levels of cholesterol for brain development and function, along with the problem of overcoming the blood–brain barrier, represent some of the many technical difficulties associated with altering cholesterol metabolism in the CNS.

Neurodegeneration in NPC disease occurs in a progressive and neuron-selective manner, and is particularly evident in Purkinje cells of the cerebellum. In NPC1-deficient mice, Purkinje cell death is first evident around 3 weeks after birth, with nearly all Purkinje cells dying by 11 weeks [75,76]. Neuronal loss is also evident in other brain regions including the substantia nigra, pons, regions of the brainstem, thalamus, and prefrontal cortex [77–79]. NPC1 deficiency in the brain causes a variety of neuronal abnormalities that could lead to neuronal dysfunction and/or death. The reason for the selective neurodegeneration in NPC disease has yet to be

elucidated. Recent experiments have shown that in *Npc1*$^{-/-}$ mice, the levels and activity of the lysosomal enzymes cathepsins B and D are elevated to a greater extent in the cerebellum than in the hippocampus which is relatively protected from neurodegeneration [80]. Furthermore, cytosolic levels of cathepsins and apoptotic molecules are higher in the cerebellum than in the hippocampus [80]. Experiments with chimeric mice, that contain both *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ cells, showed that the death of NPC1-deficient Purkinje cells was not prevented by the presence of wild-type glia [81]. Nor did NPC1-deficient glia induce death of wild-type Purkinje cells, suggesting that death of Purkinje cells is cell autonomous [81]. In support of these findings, Elrick et al. recently generated mice that lacked NPC1 in only the Purkinje cells [82]. The same extent of Purkinje cell death occurred in these mice as in NPC1 global null mice [82]. Interestingly, however, although these mice showed progressive motor impairment, they did not exhibit other typical manifestations of NPC disease such as weight loss and early death [82]. These important studies demonstrate that the premature death of NPC1-deficient mice is not due (solely) to the death of Purkinje cells.

The mechanism by which Purkinje cells, and other neurons, degenerate in NPC disease remains unclear. Markers of autophagy (beclin-1 and microtubule-associated protein 1 light chain 3-II) were detected in brains of *Npc1*$^{-/-}$ mice [83,84], and electron microscopy revealed autophagic vesicles in *Npc1*$^{-/-}$ Purkinje cells [81]. Autophagy is the process through which cells degrade long-lived proteins and organelles via lysosomes. During periods of starvation, autophagy is crucial for providing cells with energy through digestion of non-essential cellular components. Signs of autophagy have been detected in other neurodegenerative disorders including Alzheimer disease, Parkinson disease and Huntington disease, leading to speculation that excessive or impaired autophagy leads to neuronal cell death. In NPC disease, the induction of autophagy may serve as a mechanism for counteracting the abnormal accumulation of lipids within the LE/L, or as a starvation response to the sequestration of lipids in LE/L. Although it is possible that accumulation of lipids within lysosomes impairs their ability to bind to autophagosomes for degradation of engulfed material, degradation of long-lived proteins was increased in NPC1-deficient human fibroblasts, suggesting that the autophagy pathway was intact [84]. It remains to be determined whether or not autophagy contributes to neuronal death in NPC disease.

Apoptosis of neurons has also been observed in NPC disease. TUNEL-positive staining of nuclei in the cerebral cortex and cerebellum was evident in NPC patients and NPC1-deficient mice [85]. Moreover, the level of mRNAs encoding tumor necrosis factor-α and players in the tumor necrosis factor-α death pathway in the brain were higher in *Npc1*$^{-/-}$ mice than in *Npc1*$^{+/+}$ mice [85]. The expression of other markers of apoptosis, including mRNAs encoding caspases 1 and 3 [75], as well as activation of the c-Abl/p73 pro-apoptotic pathway [86], was also increased in the cerebellum of NPC1-deficient mice. Treatment of *Npc1*$^{-/-}$ mice with imatinib, an inhibitor of c-Abl, reduced Purkinje cell death, improved neurological symptoms and modestly extended the life-span [86].

In addition to neuronal death in NPC disease, other neuronal functions are likely to be impaired by NPC deficiency. The transport of cholesterol from cell bodies to distal axons of isolated neurons is reduced by NPC1 deficiency [87]. Correspondingly, the cholesterol content of cell bodies of *Npc1*$^{-/-}$ neurons is increased whereas the cholesterol content of distal axons is reduced in [69]. Moreover, the NPC1 protein is present not only in the LE/L of cell bodies of sympathetic neurons but also in recycling endosomes of pre-synaptic nerve terminals [88]. This observation might explain the defects in synaptic functions in NPC1-deficient neurons [89,90]. NPC1-deficient neurons in humans, felines, and mice, show additional abnormalities such as dendritic and axonal alterations that include ectopic dendrites and axonal spheroids [91]. Furthermore, NPC disease exhibits some similarities to Alzheimer disease as neurofibrillary tangles and hyperphosphorylation of tau are evident in both Alzheimer patients and in NPC disease patients [92,93]. However, neurofibrillary tangles are absent in NPC1-deficient mice despite the hyperphosphorylation of tau [77,94].

Although much focus has been directed at neuronal death in NPC disease, the brain also contains many glial cells which are crucial for brain function. Astrocytes, the major glial cell type in the CNS, play important roles in supporting neuronal functions, particularly at the synapse. As in other NPC1-deficient cells, *Npc1*$^{-/-}$ mouse astrocytes sequester cholesterol within LE/L [95], and reactive astrocytes have been detected throughout the brains of 4-week-old *Npc1*$^{-/-}$ mice, particularly in the cerebellum and thalamus [96]. The finding that NPC1 protein is localized predominantly in astrocytic processes in monkey brains suggests that mutations in NPC1 might impair astrocyte function [97]. Surprisingly, despite these astrocyte abnormalities, the quantity and composition of apolipoprotein E-containing lipoproteins secreted by *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mouse astrocytes are similar, and the lipoproteins secreted by astrocytes of both *Npc1* genotypes promote axon growth to the same extent [95]. On the other hand, when *Npc1*$^{+/+}$ neurons were cultured on *Npc1*$^{-/-}$ astrocytes, neurite growth was reduced compared to that of *Npc1*$^{+/+}$ neurons cultured on *Npc1*$^{+/+}$ astrocytes [98]. In addition, the expression of neurosteroid biosynthetic enzymes, and the secretion of the neurosteroid estradiol, were lower in *Npc1*$^{-/-}$ astrocytes than in *Npc1*$^{+/+}$ astrocytes [98]. Estradiol levels were also lower in NPC1-deficient mouse brains, and administration of estradiol to *Npc1*$^{-/-}$ mice alleviated some of the symptoms of NPC disease [98]. These results imply that impaired neurosteroid synthesis in NPC1-deficient astrocytes might contribute to neuronal death in NPC disease. In other experiments, NPC1 was over-expressed in astrocytes of NPC1-deficient mice using the glial fibrillary acidic protein promoter [99]. Neurodegeneration and the neuronal storage of cholesterol in the mice were decreased and life-span was increased compared to those parameters in the *Npc1*$^{-/-}$ mice [99]. However, the expression of NPC1 in astrocytes of *Npc1*$^{-/-}$ mice did not prevent Purkinje cell loss [99], suggesting that astrocyte dysfunction alone does not account for the extensive neuron loss in NPC disease.

Microglia are the resident immune cells of the CNS and play a key role in maintaining brain health. In a healthy brain, microglia exist in the "ramified" state for continuous monitoring of the brain environment [100]. Perturbation of this environment can activate microglia and lead to a variety of responses, depending on the stimulus. For example, microglia can secrete anti-inflammatory cytokines, as well as pro-inflammatory cytokines and potentially cytotoxic molecules that include glutamate and reactive oxygen species (reviewed in [101]). Increasing evidence suggests that chronic microglial activation or microglial dysfunction contributes to neuronal death in various neurodegenerative disorders including Alzheimer disease, Parkinson disease, and the lysosomal storage disorder, Sandhoff disease. In 2-week-old *Npc1*$^{-/-}$ mice, microglia with an "active" morphology were detected in brain regions such as the cerebellum, and the number of these cells increased throughout the brain as the disease progressed [96]. Moreover, the level of mRNA encoding the pro-inflammatory cytokine, tumor necrosis factor-α, in the cerebellum was increased by NPC1 deficiency [75,85]. Primary microglial cultures from NPC1-deficient mice show a punctate cholesterol staining pattern, as well as increased immunostaining for tumor necrosis factor-α (K.B. Peake and J.E. Vance, unpublished observations).

Although experiments in chimeric mice suggest that Purkinje cell death is cell autonomous [81], these experiments did not

determine if *Npc1*$^{-/-}$ Purkinje cells were more susceptible than *Npc1*$^{+/+}$ Purkinje cells to killing by *Npc1*$^{-/-}$ microglia. It is also possible that microglia contribute to the death of neurons other than Purkinje cells. Baudry et al. detected active microglia in *Npc1*$^{-/-}$ mouse brains, prior to any signs of neurodegeneration, suggesting that NPC1 deficiency in microglia might activate the microglia and, consequently, cause neuronal death [96]. It is also possible that neuronal damage and dysfunction that are not easily detected experimentally can activate microglia, which in turn might exacerbate the neurodegeneration. Conversely, NPC1 deficiency in microglia might impair normal microglial function and prevent the microglia from rescuing or repairing damaged neurons. Thus, more studies are required to determine if the microglial activation that occurs in NPC1-deficient brains contributes to, or is the result of, the neuronal death.

Oligodendrocytes are the glial cells that form myelin sheaths around axons in the brain and are, therefore, essential for proper neuronal function. The NPC1-deficient mouse brain exhibits extensive demyelination, especially in the corpus callosum, where myelin is essentially absent by 8 weeks of age [68,102]. Pre-myelinating oligodendrocytes and oligodendrocyte progenitor cells are abundant in *Npc1*$^{-/-}$ mouse brain regions that show hypomyelination, including the corpus callosum and cerebral cortex [103]. The abundance of these precursor cells and the lack of mature oligodendrocyte markers in hypomyelinated regions suggest that NPC1 deficiency causes a defect in myelination [103]. This abnormal myelination might be due to axonal damage, which occurs early during development of the NPC1-deficient mouse brain [79]. On the other hand, inclusion bodies have been detected in *Npc1*$^{-/-}$ oligodendrocytes, suggesting that defects in myelination might be due to oligodendrocyte dysfunction [104]. As is the case for oligodendrocytes in the CNS, immortalized Schwann cells of the peripheral nervous system showed punctate filipin staining [105], and hypomyelination of peripheral nerves was observed in 70-day-old *Npc1*$^{-/-}$ mice [106]. Furthermore, myelination in the peripheral nervous system of *Npc1*$^{-/-}$ mice was impaired following sciatic nerve crush injury [107]. In *Npc1*$^{+/+}$ mice, thick myelin sheaths surrounded the axons 4 weeks after the crush injury, whereas NPC1-deficient mice had thinner sheaths [107], although 10 weeks after the injury, the extent of myelination in the *Npc1*$^{-/-}$ mice was comparable to that in *Npc1*$^{+/+}$ mice [108]. These alterations in myelination might contribute to the neuronal dysfunction and degeneration characteristic of NPC disease.

Thus, the implications of NPC1 deficiency in the nervous system are very complex and incompletely understood. Experimental evidence indicates that the neurological phenotype of NPC disease, and the extensive death of neurons, likely arise from an interplay among the different cell types in the brain: neurons, astrocytes, microglia and oligodendrocytes.

5. Therapies for NPC disease

No treatments are currently available for NPC disease other than those used to alleviate the symptoms caused by disease progression. Recently, however, miglustat was approved in Europe for the treatment of adult and pediatric patients with NPC disease. Miglustat had initially been approved for treatment of patients with Gaucher disease, a lysosomal GSL storage disorder [109]. Administration of miglustat to animal models of NPC disease, including mice and cats, reduced ganglioside accumulation in the brain, delayed neurological symptoms, and increased life-span [43]. Data from initial clinical trials suggest that long-term treatment of NPC patients with miglustat is well tolerated in adult and juvenile patients, and can stabilize some of the neurological symptoms [45,46].

Another therapeutic strategy that was proposed for NPC disease was the administration of neurosteroids [110]. Cholesterol is a precursor of the neurosteroids (i.e. steroids that are synthesized in the brain) that are important for neuron growth and development. In brains of NPC1-deficient mice, levels of pregnenolone and the neurosteroid allopregnanolone (ALLO), as well as the activities of the enzymes involved in ALLO synthesis, are lower than in wild-type mice [110]. Remarkably, a single injection of ALLO in 7-day-old NPC1-deficient mice delayed the onset of neurological symptoms and increased life-span. Purkinje cell survival was also increased, while levels of GM1 and GM2 gangliosides were reduced [110]. Other studies reported that ALLO reduced microglial activation and astrocyte proliferation, and increased myelination [111,112]. At the cellular level, ALLO decreased the amount of filipin-labeled cholesterol, as well as markers of autophagy and the levels of lysosomal enzymes [112]. Although a $GABA_A$ receptor antagonist blocked the beneficial effect of ALLO on Purkinje cell survival, a stereoisomer of ALLO, *ent*-ALLO (which is not a $GABA_A$ receptor agonist) promoted neuronal survival to the same extent as ALLO [110,113]. Langmade et al. proposed that induction of pregnane X receptor-dependent gene expression by treatment of NPC1-deficient mice with ALLO or *ent*-ALLO mediated the beneficial effects of the neurosteroid [113]. However, it is difficult to rationalize why ALLO would improve the neurodegenerative phenotype through the pregnane X receptor since expression of this receptor was undetectable in the brain [74].

Recently, the reported beneficial effects of ALLO have come under intense scrutiny due to effects caused by the vehicle, cyclodextrin (CD), that was used in the experiments with ALLO described above. CD is a cyclic oligosaccharide capable of sequestering lipids, especially cholesterol, within its hydrophobic core [114]. Interestingly, recent work of Liu et al. found that treatment of NPC1-deficient mice with CD delayed the neurodegeneration and prolonged the life of the mice independent of ALLO administration [37]. A single dose of CD given to 7-day-old NPC1-deficient mice reduced the accumulation of unesterified cholesterol, increased the amount of CE, and decreased cholesterol synthesis in the liver, spleen and, to a lesser extent, the brain [115]. In addition, chronic administration of CD to mice delayed clinical onset of the disease and increased life-span of both NPC1- and NPC2-deficient mice [116]. Filipin staining and immunohistochemical analysis revealed that chronic CD treatment of mice reduced the amount of unesterified cholesterol, as well as the gangliosides GM1 and GM2, in NPC1- and NPC2-deficient mouse brains [116]. Although the mechanism underlying this striking effect of CD has not been completely elucidated, the current model is that CD enters the endocytic pathway through bulk-phase endocytosis and releases cholesterol and other lipids that are trapped in the LE/L so that the cholesterol can reach the ER [115]. In support of this model, in NPC1-deficient mouse brains and livers the levels of the mRNAs encoding sterol-response element-binding protein-2 and its target genes were decreased by CD administration, whereas the mRNAs encoding liver X receptor target genes were increased [115]. In addition, when NPC1-deficient cultured mouse fibroblasts were treated with CD in vitro, ACAT-mediated cholesterol esterification was increased, indicating that cholesterol had been released from the LE/L and transported to the ER [117]. If, on the other hand, cholesterol had been stripped from the plasma membrane by CD, one would have expected that cholesterol synthesis would have increased as compensation for the loss of cholesterol. Thus, the finding that cholesterol synthesis was decreased, not increased, by CD treatment suggests that the primary effect of CD is mobilization of cholesterol from the LE/L rather than the plasma membrane [115]. The idea that CD can extract cholesterol from the LE/L is supported by recent work indicating that dextran-conjugated CD localizes to intracellular filipin-stained organelles and reduces cholesterol accumulation

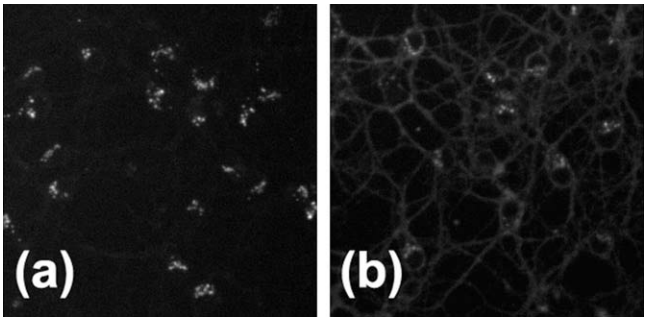

Fig. 2. CD treatment reduces the intracellular sequestration of cholesterol staining in *Npc1*$^{-/-}$ neurons. Cerebellar granule neurons, isolated from 7- to 8-day-old *Npc1*$^{-/-}$ mice, were cultured for 7 days in the absence of serum, then treated for 24 h with vehicle (a), or 0.1 mM (2-hydroxypropyl)-β-cyclodextrin (CD) (b), prior to being fixed and stained with filipin and examined by fluorescence microscopy. Images are representative of three independent experiments.

in NPC1-deficient cells [118]. Consistent with these findings, when either astrocytes or cerebellar granule neurons from *Npc1*$^{-/-}$ mice were cultured in vitro with low concentrations of CD, the punctate filipin staining was reduced (Fig. 2) and cholesterol synthesis was decreased. In contrast, higher concentrations of CD, which presumably would have extracted significant amounts of cholesterol from the plasma membrane, increased cholesterol synthesis in *Npc1*$^{-/-}$ neurons (K.B. Peake and J.E. Vance, unpublished observations). The precise mechanism by which CD mediates these effects is unknown. Other experiments by Davidson et al. suggest that the administration of ALLO alone to NPC1-deficient mice, without CD has no beneficial effects, whereas the administration of CD with ALLO is more beneficial than CD alone [116], although these conclusions remain controversial. Thus, further investigations are needed to clarify the role of ALLO in the treatment of NPC disease.

Although the recent development of potential therapies for NPC disease has shown promising advances, one of the major obstacles that remains is the efficient and rapid diagnosis of NPC disease which would allow treatment to be initiated as early as possible. NPC disease has a significant delay in diagnosis after onset of symptoms, with one recent study finding that the average delay in diagnosis was 4.3 years with a range of 3 months to 19 years [119]. Most of the experiments in which therapeutic compounds were administered to animals were performed prior to the onset of neurological symptoms. While these treatments yielded encouraging results, when the same compounds were administered at later stages of disease progression, the results were not as positive. For example, significant improvements occurred when CD was administered to 7-day-old mice prior to the onset of overt neurodegenerative symptoms. However, when a single dose of CD was given to 49-day-old mice, the amount of cholesterol that accumulated in tissues throughout the body was reduced but life-span was not extended [120]. The latter observation might be explained by the fact that CD can enter the brain from the circulation in 7-day-old mice, prior to formation of the blood–brain barrier, whereas access of CD to the brain might be impeded in adult mice with an intact blood–brain barrier. In addition, beneficial effects of CD might occur only if CD were chronically administered, particularly at later stages of disease progression. However, it appears that there might be a critical therapeutic window, after which irreversible damage occurs, so that any therapy for the disease will only prevent further progression. Thus, better screening processes and increased awareness of NPC disease are needed, as early treatment will likely have the greatest benefit. Nonetheless, recent therapeutic advances such as treatments with miglustat and CD provide options that show promise in slowing the progression of NPC disease. Due to the complex nature of NPC disease, a combination of various treatments will probably have the greatest therapeutic effect.

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