

*Thematic Review Series: Recent Advances in the Treatment of Lysosomal Storage Diseases*

## Niemann-Pick C disease and mobilization of lysosomal cholesterol by cyclodextrin

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**Abstract** Niemann-Pick type C (NPC) disease is a lysosomal storage disease in which endocytosed cholesterol becomes sequestered in late endosomes/lysosomes (LEs/Ls) because of mutations in either the *NPC1* or *NPC2* gene. Mutations in either of these genes can lead to impaired functions of the NPC1 or NPC2 proteins and progressive neurodegeneration as well as liver and lung disease. NPC1 is a polytopic protein of the LE/L limiting membrane, whereas NPC2 is a soluble protein in the LE/L lumen. These two proteins act in tandem and promote the export of cholesterol from LEs/Ls. Consequently, a defect in either NPC1 or NPC2 causes cholesterol accumulation in LEs/Ls. In this review, we summarize the molecular mechanisms leading to NPC disease, particularly in the CNS. Recent exciting data on the mechanism by which the cholesterol-sequestering agent cyclodextrin can bypass the functions of NPC1 and NPC2 in the LEs/Ls, and mobilize cholesterol from LEs/Ls, will be highlighted. Moreover, the possible use of cyclodextrin as a valuable therapeutic agent for treatment of NPC patients will be considered.—Vance, J. E., and B. Karten. Niemann-Pick C disease and mobilization of lysosomal cholesterol by cyclodextrin. *J. Lipid Res.* 2014. 55: 1609–1621.

**Supplementary key words** neurodegenerative disease • lysosomal storage disease • Purkinje cells • gangliosides • neurons • astrocytes • microglia • cholesterol homeostasis • endoplasmic reticulum

Niemann-Pick type C (NPC) (1) disease is a progressive inherited disease in which unesterified cholesterol and other lipids accumulate in late endosomes/lysosomes (LEs/Ls) of all cells and tissues. The underlying cause of NPC disease is distinct from that of Niemann-Pick disease types A/B, which are caused by defects in the lysosomal acid sphingomyelinase gene (1). NPC disease is a recessive, autosomal disorder that affects ~1/150,000 live births and causes premature death due to relentless

neurodegeneration as well as lung and liver dysfunction (reviewed in Refs. 2, 3). Because neuropathological changes in NPC disease start long before the onset of symptoms, early diagnosis of the disease is crucial. However, clinical diagnosis of NPC disease in childhood can be difficult as the early symptoms are typical of many other neurological disorders, and therapeutic intervention is largely restricted to symptomatic treatment. Furthermore, access of therapeutic agents to the CNS is often problematic because the blood-brain barrier is impermeable to many molecules and prevents their passage into the brain from the peripheral circulation. In this review, we will summarize the molecular mechanisms that lead to NPC disease and discuss recent exciting data indicating that the cholesterol-sequestering agent, cyclodextrin, might be a valuable therapeutic agent for treatment of NPC disease.

### BIOCHEMICAL AND CELL BIOLOGICAL IMPLICATIONS OF NPC DEFICIENCY

In mammalian cells, cholesterol is a key component of cellular membranes and is a precursor of steroid hormones and bile acids. Cells acquire cholesterol from endogenous synthesis in the endoplasmic reticulum (ER) and from receptor-mediated uptake of exogenous LDLs via LDL receptors (4). The underlying cellular defect in NPC disease is an impaired egress of endocytosed, LDL-derived unesterified cholesterol from LEs/Ls, resulting in the accumulation of unesterified cholesterol in LEs/Ls (5). This cellular defect was first revealed by Pentchev and

Abbreviations: CYCLO, 2-hydroxypropyl- $\beta$ -cyclodextrin; ER, endoplasmic reticulum; HDAC, histone deacetylase; LE/L, late endosome/lysosome; NPC, Niemann-Pick type C; SREBP, sterol response element-binding protein.

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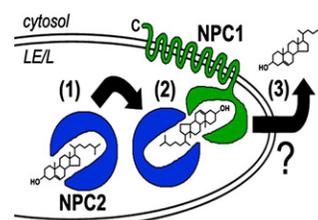
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coworkers (6) who demonstrated that the esterification of LDL-derived cholesterol was profoundly diminished in fibroblasts from NPC patients. However, the activity of the cholesterol esterification enzyme, ACAT, as well as the receptor-mediated uptake of LDL and the lysosomal hydrolysis of LDL-derived cholesteryl esters were normal. Thus, these observations indicated that LDL-derived cholesterol was sequestered in LEs/Ls because export of unesterified cholesterol from LEs/Ls was disrupted. Consequently, the amount of cholesterol reaching the ER and plasma membrane from LEs/Ls was reduced (6–9). In contrast, the transport of newly synthesized cholesterol from the ER to the plasma membrane occurs normally in fibroblasts from NPC patients (8, 10).

Homozygous mutations in either the *NPC1* gene (11, 12) or the *NPC2* gene (13, 14) were subsequently identified as being causative for NPC disease. Most (~95%) identified cases of NPC disease are due to mutations in the *NPC1* gene, whereas the remaining 5% of cases are caused by mutations in the *NPC2* gene. The *NPC1* gene encodes an LE/L membrane protein that contains 1,254 amino acids, 13 presumed transmembrane domains, a leucine zipper, a lysosomal targeting motif, and a sterol-sensing motif (12). The latter motif is similar to a sequence that has been identified in several proteins involved in cholesterol metabolism. The *NPC2* gene (originally called *HE1*) encodes a 151-amino-acid soluble protein of the LE/L lumen (14, 15). The NPC1 and NPC2 proteins both bind cholesterol (16–19). The N-terminal domain of NPC1 is exposed to the LE/L lumen and binds cholesterol with high affinity in a 1:1 molar ratio (18–22). Fluorescence dequenching assays show that NPC2 rapidly transfers cholesterol, but not glycosphingolipids, ceramide, phospholipids, or fatty acids (16, 19, 21), between phospholipid liposomes in vitro. This transfer is promoted by an acidic environment and is markedly stimulated by the presence of bis(monoacylglycerol) phosphate (16, 23), a phospholipid that is highly enriched in luminal multivesicular bodies of LEs/Ls and accumulates in NPC-deficient cells (24). X-ray crystallographic studies showed that orientation of the cholesterol molecule attached to NPC2 is opposite from that of cholesterol bound to the hydrophobic pocket of NPC1 (17, 18, 20–22).

Importantly, homozygous mutations in either *NPC1* or *NPC2* cause essentially the same clinical and cellular phenotypes (25, 26). Thus, a model was proposed for the concerted transfer of cholesterol from NPC2 to NPC1 within LEs/Ls (Fig. 1). In this model, endocytosed unesterified cholesterol is delivered to LEs/Ls and then transferred via NPC2 from membranes of the internal vesicles of LEs/Ls to NPC1 in the limiting membrane (19). An important feature of this model is that the hydrophobic cholesterol molecule can be exported without direct contact with the aqueous lumen of LEs/Ls. In support of this model, a direct interaction between NPC2 and a luminal domain of NPC1 was demonstrated (27). This protein-protein interaction occurs only at acidic pH similar to that of the LE/L lumen and requires that cholesterol be bound to NPC2. The molecular mechanisms by which cholesterol is



**Fig. 1.** Proposed mechanism for the concerted action of NPC1/NPC2 in cholesterol export from LEs/Ls. NPC2 (blue), a soluble protein in the LE/L lumen, binds endocytosed cholesterol in the LE/L lumen (1). Subsequently, NPC2 shuttles the cholesterol to the cholesterol-binding pocket of NPC1 (green), a polytopic protein in the LE/L limiting membrane, without direct contact of the hydrophobic cholesterol molecule with the aqueous LE/L lumen (2). The mechanism by which NPC1-associated cholesterol is exported from the outer LE/L membrane to the ER and plasma membrane (3) has not been established. Reproduced from Ref. 165 with permission.

exported from NPC1 on the LE/L limiting membrane to the ER and plasma membrane have not yet been elucidated, although vesicular transport involving Rab proteins (28, 29), transport via carrier proteins such as oxysterol binding proteins (30, 31), and direct membrane contact have been proposed.

Because the sequestration of cholesterol in LEs/Ls of NPC-deficient cells impairs cholesterol delivery to the ER (6–8), the amount of cholesterol in the ER, as a molar % of total ER lipids, can fall below a critical threshold (32). Thus, although cholesterol accumulates in LEs/Ls, there is a deficit of cholesterol in the ER so that the sterol response element-binding protein (SREBP) pathway (33) is enhanced, and the synthesis and uptake of cholesterol are increased (9, 34). The impaired trafficking of cholesterol in NPC-deficient cells profoundly affects multiple cellular functions such as regulation of lysosomal calcium homeostasis (35), oxidative stress (36, 37), and vesicle trafficking pathways mediated by Rab proteins (28, 29), as well as fusion of LEs/Ls (38). NPC1 deficiency is particularly detrimental for the functioning of LEs/Ls in the brain (39). Intriguingly, cholesterol transport from LEs/Ls to mitochondria is not defective in NPC1-deficient cells (40). Indeed, the cholesterol content of mitochondria isolated from brains is increased, not decreased, by NPC1 deficiency (40, 41). Several properties of mitochondria such as ATP production, oxidative stress, and possibly mitophagy are altered by NPC1 deficiency (41–43), probably because of the increase in mitochondrial cholesterol. In contrast to deficiency of NPC1, however, the movement of endosomal cholesterol to mitochondria is disrupted by NPC2 deficiency (44). Moreover, mutants of NPC2 that bind cholesterol, but cannot transfer cholesterol to NPC1, can restore cholesterol trafficking to mitochondria in NPC2-deficient cells (44). Because NPC2 transfers cholesterol directly to membranes (23), a possible explanation for this difference between NPC1 and NPC2 is that NPC2 transfers cholesterol from the LE/L lumen directly to the perimeter membrane of LEs/Ls, as well as to transmembrane proteins such as MLN64 and NPC1.

## CLINICAL MANIFESTATIONS OF NPC DEFICIENCY

The clinical manifestations of NPC disease usually become apparent in early childhood, but the age of onset and rate of disease progression vary over a wide range (2, 45, 46). In both humans and mice, the most severe consequence of NPC deficiency is neurodegeneration, particularly of Purkinje neurons in the cerebellum, resulting in progressive motor impairment (47, 48). In other brain regions, neuronal abnormalities, such as axonal swelling and degeneration and ectopic dendrite formation, also occur and are often more prominent than neuron death (49–52). These impairments contribute to the reduced weight gain, cognitive decline, and premature death that characterize NPC disease. Severe liver disease is also prevalent in many NPC-deficient patients (2) and mice (53–56). The majority (~80%) of circulating LDLs are cleared by the liver via receptor-mediated endocytosis (57, 58). Consequently, cholesterol accumulation in the LEs/Ls of NPC1-deficient patients and mice is more pronounced in liver than in any other tissue (58, 59). The lung is another tissue whose function is impaired in NPC disease (58, 60–62), and lipid-laden macrophages are abundant in the lungs of NPC-deficient mice (63). Moreover, age-related retinal degeneration occurs in *Npc1*<sup>-/-</sup> mice (64). Two recent studies have reported that levels of mRNAs and proteins of several enzymes of energy metabolism are altered and that lactate levels are increased in NPC1-deficient cerebella. Moreover, pyruvate oxidation is decreased and anaerobic glycolysis is increased during progression to the symptomatic stage of NPC disease (65, 66). Thus, glucose metabolism appears to be affected at early stages of NPC disease. Markers of oxidative stress are also increased in brains of human NPC patients and animal models (36, 37, 67).

## MODELS USED FOR STUDYING NPC DISEASE

Several cell and animal models of NPC disease are available and have provided key information on the mechanism by which deficiency of NPC1 or NPC2 leads to the disease phenotype. The cell biological and biochemical defects in NPC-deficient cells were initially discovered using NPC1-deficient fibroblasts (mutant Chinese hamster ovary cells and fibroblasts from NPC patients) (6, 7, 10, 34, 68, 69). Although the class II amphiphilic amine, U18666, also causes cholesterol sequestration in LEs/Ls and has been widely used to mimic the NPC defect in cultured cells, this drug produces side effects including inhibition of cholesterol synthesis (70, 71). The most widely used model of NPC disease is the Balb/cNctr-*Npc*<sup>N/+</sup> mouse that completely lacks NPC1 protein (72). These mice are asymptomatic at birth but develop ataxia and die within ~3 months. A more recent mouse model carries the D1005G mutation in NPC1 (73). This mutation is in the cysteine-rich loop of NPC1, the site at which the most common mutations occur in human NPC patients, and these mice have a more slowly progressing disease than that of NPC1-null

mice (73). NPC1 expression has also been reduced in mouse liver by antisense technology (74). Hypomorphic NPC2-deficient mice (that express only 0.4% of the normal amount of NPC2 protein) (26) and NPC2-null mice (75) have also been generated. The phenotype of these mice is similar to, but slightly less severe than, that of *Npc1*<sup>-/-</sup> mice (26, 75). More recently, NPC1 was eliminated specifically in certain cell types (76) and, reciprocally, was expressed only in specific cells of *Npc1*<sup>-/-</sup> mice (77). These studies have been particularly useful for understanding in which cells of the brain loss of NPC function is the most detrimental (see Loss of NPC Function in Cells of the Brain). As an alternative to mouse models of NPC disease, an NPC1-deficient feline model was established (78, 79) and has provided valuable information relevant to human NPC disease. Other models that have been used to elucidate pathways defective in NPC disease are mutants of yeast (80) and *Drosophila* (81, 82). An advantage of these nonmammalian models is that genetic manipulation is relatively straightforward. Recently, studies have been initiated in human stem cells isolated from individual NPC patients, particularly stem cells that were differentiated into neuron-like cells (43, 83–85). It is likely that these cells will be useful for evaluation of drug candidates for NPC disease.

## LIPID ACCUMULATION IN THE LEs/Ls

Cholesterol is not the only lipid that accumulates abnormally in LEs/Ls of NPC-deficient cells. Sphingomyelin (86), gangliosides (particularly GM2 and GM3) (87–89), sphingosine (35), and bis(monoacylglycerol) phosphate (24, 90) also accumulate. Compelling evidence indicates that the primary offending molecule is cholesterol and that the accumulation of the other lipids is secondary to the sequestration of cholesterol. First, NPC1 and NPC2 each bind cholesterol with high affinity, but not gangliosides or sphingosine (17, 18, 91). In addition, a mutation in the cholesterol-binding site of NPC1 abolished the binding of cholesterol, and expression of this mutant protein in mice induced the typical NPC phenotype (92). Second, the storage of gangliosides was genetically reduced by crossing *Npc1*<sup>-/-</sup> mice with mice that lacked the galactosyltransferase responsible for synthesis of GM2 ganglioside or with mice lacking GM3 synthase (93). In these mice, ganglioside storage in LEs/Ls was eliminated, but cholesterol still accumulated and the neurodegeneration was not improved (93–95). Third, because lysosomal acid sphingomyelinase activity is inhibited when the amount of cholesterol in LE/L membranes is increased, the accumulation of sphingomyelin in LEs/Ls of NPC-deficient cells also appears to be secondary to cholesterol sequestration (86, 96). Fourth, although sphingosine accumulation was reported prior to cholesterol accumulation (35), these studies were not performed in NPC1-deficient cells but in cells treated with the amphiphile U18666A, which also inhibits cholesterol synthesis. Fifth, 2-hydroxypropyl- $\beta$ -cyclodextrin

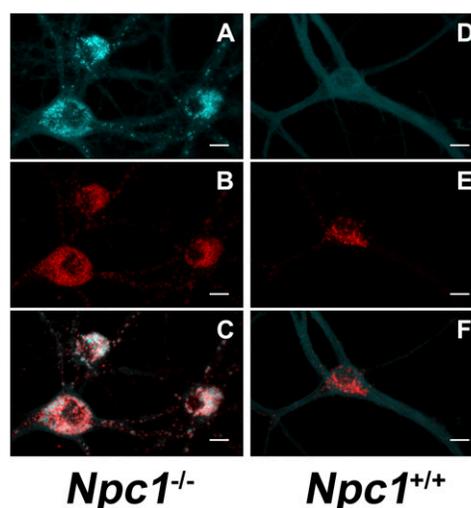
(CYCLO), which forms an inclusion complex with cholesterol (reviewed in Ref. 97), dramatically reduced cholesterol storage in tissues of NPC-deficient mice, thereby attenuating the neurodegeneration and extending life span (98–100) (see Cyclodextrin as a Therapy for NPC Disease). These data provide convincing evidence that cholesterol sequestration in LEs/Ls of NPC-deficient cells is the primary event responsible for NPC disease.

#### LOSS OF NPC FUNCTION IN CELLS OF THE BRAIN

Although NPC1 and NPC2 appear to be expressed in all cells and tissues, the most devastating consequences of NPC deficiency typically occur in the brain. Cholesterol metabolism in the brain is compartmentalized from that in the periphery by the blood-brain barrier (101, 102). Thus, essentially all cholesterol in the brain is synthesized within the brain rather than being delivered from plasma lipoproteins. The most striking consequence of NPC1 deficiency in the brains of mice and humans is a profound loss of Purkinje neurons in the cerebellum (103–105). In other regions of the brain, such as the cerebral cortex and thalamus, neuronal apoptosis also occurs but is less common than axonal swelling and ectopic dendrite formation (51, 52, 106–109). The reason why Purkinje neurons are the most sensitive neurons to NPC deficiency is not clear (39).

Interestingly, although cholesterol accumulates in NPC1-deficient peripheral tissues, such as the liver, the cholesterol content of NPC-deficient brains does not increase, but even decreases, with age. The reason for this apparent anomaly is that myelin contains ~75% of cholesterol in the brain, and extensive demyelination occurs in NPC-deficient brains (58, 110). In addition, genetic deletion of NPC1 in oligodendrocytes of mice reduces myelin formation (111, 112). Cholesterol does, however, accumulate in LEs/Ls of NPC-deficient neurons, such as *Npc1*<sup>-/-</sup> mouse sympathetic neurons (113), hippocampal neurons (Fig. 2) (114), and cerebellar granule neurons (115). In *Npc1*<sup>-/-</sup> sympathetic neurons, cholesterol accumulates in LEs/Ls of the cell bodies, whereas the cholesterol content of distal axons is less than that of *Npc1*<sup>+/+</sup> neurons (113). In addition, cholesterol transport from cell bodies to distal axons is impaired by lack of NPC1 (116). Although the accumulation of cholesterol in cell bodies of NPC-deficient neurons is generally considered to be the major cause of neurodegeneration, it is also possible that a deficiency of cholesterol in distal axons contributes to the neurological phenotype.

The NPC1 protein is present in recycling endosomes of presynaptic nerve terminals, and the morphology of synaptic vesicles isolated from brains of *Npc1*<sup>-/-</sup> mice is aberrant (117). Moreover, defects in synaptic transmission have been observed in several brain areas and in experimental models of NPC1 deficiency. Synaptic membranes are relatively rich in cholesterol, which is required for synaptic transmission at many levels. For example, the activity of pre- and postsynaptic receptors is influenced by membrane cholesterol content (reviewed in Refs. 118, 119). In



**Fig. 2.** Cholesterol sequestration in LEs/Ls of *Npc1*-deficient neurons. Primary hippocampal neurons from E17 *Npc1*<sup>-/-</sup> (A–C) and *Npc1*<sup>+/+</sup> (D–F) mice were cultured for 14 days in serum-free medium and then stained for cholesterol (filipin) (A, D) and with antibodies directed against lysosome-associated membrane protein-1, LAMP1 (B, E); merge (C, F). Scale bars indicate 20  $\mu$ m.

addition, the formation of presynaptic SNARE complexes, the interaction between synaptobrevin and synaptophysin, the fusion of synaptic vesicles with presynaptic membranes, and the sorting of synaptic vesicles during endocytosis all depend on cholesterol (120–124). Thus, extraction of cholesterol from neurons, by brief incubation with cyclodextrin, impaired exocytosis and endocytosis of synaptic vesicles (114, 125–128). Moreover, defects in exocytosis and mobilization of synaptic vesicles, as well as increased spontaneous neurotransmission, were observed in cultured hippocampal neurons from *Npc1*<sup>-/-</sup> mice (114, 126, 129). The inhibitory GABAergic synapses were more severely affected than glutamatergic synapses, indicating an imbalance between inhibitory and excitatory neurotransmission (129). Studies in hippocampal slices also revealed hyperexcitability of the neuronal network in NPC1-deficient brains (114). Furthermore, NPC1 deficiency alters long-term synaptic plasticity in several brain regions (129–132). The presynaptic defects in NPC1-deficient neurons are independent of the presence of glial cells (133).

The mechanism by which defective export of cholesterol from LEs/Ls leads to neuropathology is poorly understood. Thus, an important question is, in which cell type(s) of the brain is NPC deficiency responsible for the neurological deficits? The three major types of cells in the brain are neurons, astrocytes, and microglia, all of which sequester cholesterol in LEs/Ls in response to NPC deficiency (113, 115, 134, 135). Clearly, inflammation is widespread in NPC-deficient brains. Microglia are resident immune cells of the CNS and proliferate and become activated in response to changes in their environment. Microglia participate in phagocytosis of injured/dying cells (136) and can generate a protective inflammatory response (137, 138). On the other hand, chronic activation of microglia can induce neuroinflammation and neuronal death in several neurodegenerative disorders (reviewed in

Ref. 139). Microglial proliferation and activation are rampant in specific brain regions of NPC disease patients and *Npc1*<sup>-/-</sup> mice, prior to onset of neurological symptoms (50, 52, 135). Thus, the microglial activation that occurs in NPC disease could either be a protective mechanism induced in response to neuronal death or could contribute directly to neuronal death. In mouse brains, NPC1 deficiency increased expression of mRNAs encoding proinflammatory cytokines, such as tumor necrosis factor (105, 108, 140). Moreover, treatment of *Npc1*<sup>-/-</sup> mice with a liver X receptor agonist reduced microglial activation and neuroinflammation and prolonged survival of the mice (140). However, this agonist alters the expression of genes involved in multiple pathways, and one cannot conclude that the decrease in neuroinflammation was directly linked to the increased life span. The majority of recent data indicate that inflammation in NPC-deficient brains is primarily a response to the neurodegeneration. For example, when NPC1 was eliminated *only* in neurons of mice, neuropathology developed typical of that in mice in which NPC1 was eliminated globally (141). Moreover, expression of NPC1 only in neurons of *Npc1*<sup>-/-</sup> mice prevented the neurodegeneration (77). In addition, in microglia-neuron cocultures, the presence of activated *Npc1*<sup>-/-</sup> microglia did not cause neuron death (135). Consistent with the idea that inflammation is secondary to neuron death, deletion of the macrophage inflammatory gene *Mip1a/Ccl3* in *Npc1*<sup>-/-</sup> mice did not prevent neurodegeneration (142), nor did deletion of the complement system in *Npc1*<sup>-/-</sup> mice reduce neuronal death or prolong survival (143). In combination, these experiments imply that microglial activation in NPC-deficient brains is primarily a response to neuronal death, rather than a direct cause of the neurodegeneration.

The proliferation and activation of astrocytes are also widespread in NPC1-deficient brains (50). However, this astrogliosis does not appear to be the primary cause of the neurodegeneration. For example, in a hybrid mouse model, *Npc1*<sup>+/+</sup> neurons were not killed by surrounding *Npc1*<sup>-/-</sup> astrocytes/microglia (144). Furthermore, genetic deletion of NPC1 in astrocytes alone did not cause the neuropathology exhibited by *Npc1*<sup>-/-</sup> mice (141). Nor did Tet-induced expression of NPC1 in astrocytes alone of *Npc1*<sup>-/-</sup> mice slow disease progression, except for a small delay in weight loss (77). In contrast, one study reported that cholesterol storage was decreased, and survival of *Npc1*<sup>-/-</sup> mice was increased, by expression of NPC1 in astrocytes under control of the glial fibrillary acidic protein promoter. However, NPC1 expression in other cell types was not completely excluded (145). On the other hand, neuron-specific deletion of NPC1 caused activation of astrocytes and microglia in the murine brain (141, 143). Consequently, the astrogliosis and inflammation in NPC-deficient brains appear to be largely secondary to NPC deficiency in neurons.

Overall, these observations in NPC-deficient cells of the brain strongly imply that the absence of NPC1 in neurons alone is sufficient to cause the neuropathology. Consistent with this conclusion, NPC1 expression only in neurons of

*Npc1*<sup>-/-</sup> mice prevented the neurodegeneration (77, 146). More specifically, expression of NPC1 in cerebellar Purkinje neurons (the neurons most affected in NPC disease), but not in neurons of other forebrain areas, of *Npc1*<sup>-/-</sup> mice markedly extended life span and lessened the ataxia (77). In addition, elimination of NPC1 specifically in Purkinje neurons caused ataxia and loss of Purkinje cells in an anterior-to-posterior gradient, but, surprisingly, these mice did not exhibit weight loss or premature death (76). In another study, NPC1 was eliminated from neurons in nearly all brain areas, although NPC1 was still expressed in Purkinje cells, yet the mice exhibited the same neurodegenerative phenotype as that of NPC1-null mice (141). Thus, although lack of NPC1 in Purkinje cells contributes to Purkinje cell death and the neuropathology in NPC disease, lack of NPC1 in other types of neurons is clearly also detrimental. Overall, these observations demonstrate that NPC deficiency in neurons, rather than in astrocytes or microglia, is the primary cause of neuropathology in NPC disease.

#### CYCLODEXTRIN AS A THERAPY FOR NPC DISEASE

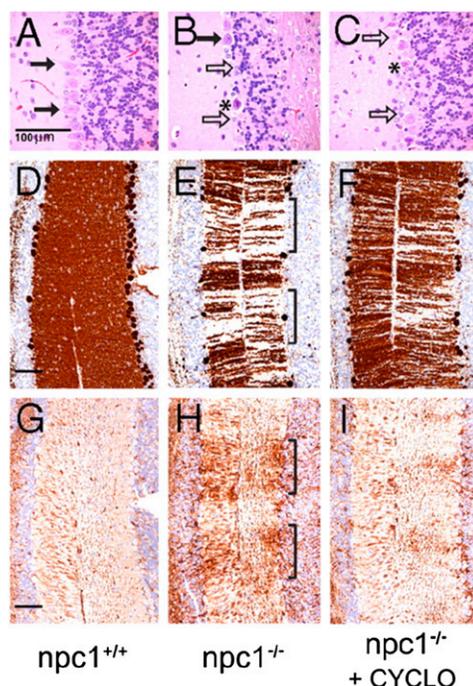
Potential therapies for NPC disease have focused on reduction of lipid storage in LEs/Ls. An important consideration when testing therapies for NPC disease patients is how disease progression can be quantitatively assessed. Thus, validation of biomarkers of NPC disease progression is an important goal (147–149). Because gangliosides accumulate in LEs/Ls of NPC-deficient cells, miglustat, which efficiently crosses the blood-brain barrier, has been used for treatment of NPC patients. Miglustat inhibits glucosylceramide synthase, a key enzyme of glycosphingolipid synthesis (reviewed in Ref. 150). In the feline model of NPC disease, miglustat decreased GM2 ganglioside accumulation and slightly delayed development of neurological symptoms (151). Similarly, in *Npc1*<sup>-/-</sup> mice, miglustat reduced ganglioside storage in LEs/Ls. In addition, miglustat modestly extended life span of (87) and produced some neurological improvements in NPC patients (152–156). However, the mechanism underlying these clinical improvements is not clear because reduction of gangliosides in LEs/Ls of NPC-deficient mice does not appear to be beneficial (93–95).

Several other treatments for NPC disease are currently being investigated (reviewed in Ref. 157). One of the most promising is inhibition of histone deacetylases (HDACs). HDACs appear to play important roles in neuronal survival (158, 159). HDAC inhibitors have already been approved for clinical use in humans for treatment of certain cancers. A clinically approved HDAC inhibitor decreased HDAC expression in NPC1-deficient cells and reduced cholesterol accumulation in LEs/Ls (160, 161). Interestingly, however, treatment of NPC2-deficient human fibroblasts with an HDAC inhibitor did not reduce cholesterol storage in LEs/Ls (161). The expression of several HDAC genes is increased by NPC deficiency in human fibroblasts

(160, 161). Because HDAC inhibitors increase the amount of NPC1 protein (162), these inhibitors might be useful in NPC patients in which residual NPC1 activity remains or where partially active NPC1 protein is mislocalized (163). The antiapoptotic agent imatinib has also been suggested as an agent that might prevent neurodegeneration in NPC disease by inhibiting the c-Abl pathway, the activity of which is increased in *Npc1*<sup>-/-</sup> Purkinje cells (164). Imatinib treatment of *Npc1*<sup>-/-</sup> mice partially prevented Purkinje cell death and modestly improved neurological parameters, but only slightly prolonged life (164).

A very exciting approach toward treatment of NPC disease is based on the discovery that the cholesterol-binding agent CYCLO markedly delays neurodegeneration and increases life span of *Npc1*<sup>-/-</sup> mice [(95, 98, 99); reviewed in Ref. 165]. These studies were prompted by a report that in 7-day-old *Npc1*<sup>-/-</sup> mice injection of the neurosteroid allopregnanolone, dissolved in CYCLO, delayed the neurodegeneration and approximately doubled life span (166). Subsequent experiments revealed, however, that injection of allopregnanolone alone, without CYCLO, into 7-day-old *Npc1*<sup>-/-</sup> mice did not produce the beneficial effects (98). On the other hand, a single intraperitoneal injection of CYCLO (4,000 mg/kg body weight), the carrier of the allopregnanolone (166), did reduce the neurodegeneration and increased life span by ~50% (95, 98). Thus, the reported beneficial effects of allopregnanolone in *Npc1*<sup>-/-</sup> mice (166) appear to be attributable to CYCLO. Additional benefits are gained by repeated administrations of CYCLO. For example, subcutaneous injection of CYCLO (4,000 mg/kg) into *Npc1*<sup>-/-</sup> mice once per week for 7 weeks reduced cholesterol accumulation in most tissues, markedly improved neurodegeneration and liver function, and approximately doubled life span (60), although the pulmonary disease was resistant to CYCLO (60). When CYCLO (4,000 mg/kg) was subcutaneously injected into *Npc1*<sup>-/-</sup> mice on alternate days, starting at either day 7 or day 21 after birth, the neuronal storage of cholesterol and gangliosides was reduced, neurodegeneration was delayed, and life span was prolonged (99). Importantly, injection of CYCLO into NPC2-deficient mice was similarly beneficial (99, 167). In careful "balance" studies on quantification of the cholesterol excreted into bile by *Npc1*<sup>-/-</sup> mice, CYCLO reduced cholesterol storage in almost all tissues and promoted the excretion of cholesterol-derived bile acids (100). Importantly, although CYCLO injection mobilized LE/L cholesterol and reduced inflammatory parameters in 49-day-old *Npc1*<sup>-/-</sup> mice (Fig. 3), their life span was not increased, probably because extensive tissue damage had already occurred (100). Moreover, CYCLO is cleared from the body 6 times more rapidly in mature mice than in 7-day-old *Npc1*<sup>-/-</sup> pups, which could further contribute to the lower efficacy of CYCLO in older mice (100).

The studies described previously on the remarkable effects of subcutaneous injection of CYCLO into 7-day-old mice (Fig. 3) (95, 98, 99) imply that at least some CYCLO crosses the blood-brain barrier and enters the CNS. Nevertheless, a recent study concluded that the amount of



**Fig. 3.** CYCLO prevents Purkinje cells loss in *Npc1*<sup>-/-</sup> mice. *Npc1*<sup>-/-</sup> (B, C, E, F, H, I) and *Npc1*<sup>+/+</sup> (A, D, G) mice (7 days old) were given a single intraperitoneal injection of CYCLO (4,000 mg/kg body weight). At 49 days of age, sections of the cerebellum were stained with hematoxylin and eosin (A–C), for calbindin (Purkinje cell marker) (D–F), and for glial fibrillary acidic protein (marker of astrocyte activation) (G–I). Scale bars indicate 100  $\mu$ m. Arrows indicate loss of Purkinje cells in a band-like pattern (indicated by brackets) in untreated *Npc1*<sup>-/-</sup> mice; \*, necrotic Purkinje cells. Reproduced from Ref. 98 with permission.

CYCLO that crosses the blood-brain barrier from plasma in mature mice is very small, and that the neurological benefits of peripherally injected CYCLO are the result of CYCLO binding to the cerebral vascular endothelium (168). Other studies, however, have indicated that a small percentage of CYCLO in the peripheral circulation does cross the blood-brain barrier (169); it is also possible that in 7-day-old, but not older, mice the blood-brain barrier is incompletely closed and is partially permeable to CYCLO. In light of the poor permeability of the blood-brain barrier to CYCLO, CYCLO was injected directly into the CNS of *Npc1*<sup>-/-</sup> mice. The CYCLO diffused throughout all areas of the brain, and the benefits lasted more than a week (170). CYCLO was, therefore, infused continuously by intracerebroventricular delivery (35 mg/kg/day) into brains of *Npc1*<sup>-/-</sup> mice for 4 weeks, starting at 3 weeks of age. When this treatment was combined with weekly subcutaneous injections of CYCLO (4,000 mg/kg), the histology of all regions of the brain was normal and neurodegeneration was eliminated (170). Importantly, in *Npc1*<sup>+/+</sup> mice, cholesterol homeostasis was not altered when CYCLO was delivered peripherally or intracerebroventricularly (170). The ED<sub>50</sub> for the therapeutic effect of CYCLO is 0.5 mg/kg (100, 170). The concentration of CYCLO required to mobilize LE/L cholesterol in NPC1-deficient cells in either peripheral tissues or in the CNS of *Npc1*<sup>-/-</sup> mice is <1 mM,

probably  $\sim 0.1$  mM (100, 115, 170). No toxic effects of these low doses of CYCLO were evident in *Npc1*<sup>-/-</sup> mice, and CYCLO has been approved by the Federal Drug Administration (USA) as a drug delivery vehicle in humans. These exciting studies suggest that the simultaneous delivery of CYCLO by subcutaneous injection into the periphery and by direct infusion into the CNS might represent a valid, if cumbersome, therapeutic approach for treatment of NPC patients. Indeed, a clinical trial in which CYCLO is being delivered intrathecally via osmotic minipumps to a few NPC patients was initiated at the National Institutes of Health (USA) in 2013; the outcome is anxiously awaited.

The effectiveness of CYCLO was also evaluated in the feline NPC model. As in NPC-deficient mice, CYCLO (4,000 mg/kg) markedly attenuated the neurodegeneration and liver disease in the cats (79). However, a dose-dependent hearing defect developed in the CYCLO-treated cats suggests that CYCLO damages the peripheral auditory pathway (79). This unexpected side effect was further investigated by administration of a single dose of CYCLO subcutaneously (8,000 mg/kg), or intrathecally (120 mg), to *Npc1*<sup>+/+</sup> cats. The cats exhibited a significant increase in hearing threshold. However, the mechanism by which CYCLO caused this effect has not been established (79). Consequently, it will be important to determine whether CYCLO causes a hearing defect in NPC patients.

#### MECHANISM BY WHICH CYCLO IMPROVES THE NPC PHENOTYPE

Cyclodextrins, such as CYCLO, are cyclic oligosaccharides consisting of multiple glycopyranose units (reviewed in Ref. 97) and have been used for delivery of hydrophobic drugs to humans. The cyclodextrins are water soluble but contain a hydrophobic pocket. Cyclodextrins typically exist as hexamers ( $\alpha$ -cyclodextrins), heptamers ( $\beta$ -cyclodextrins), or octamers ( $\gamma$ -cyclodextrins), which contain hydrophobic cavities of different sizes. The  $\beta$ -cyclodextrins have the highest affinity for cholesterol and are the most efficient in extracting cholesterol from cells. In contrast,  $\alpha$ -cyclodextrins are the most effective at extracting phospholipids from cells: the hydrophobic cavity of  $\alpha$ -cyclodextrins appears to be too small to accommodate cholesterol. The binding pocket of  $\gamma$ -cyclodextrins is less hydrophobic than that of  $\beta$ -cyclodextrins. The water solubility of  $\beta$ -cyclodextrins can be increased by modification of the hydrophilic groups with moieties such as 2-hydroxypropyl groups. The methyl- and 2-hydroxypropyl derivatives of  $\beta$ -cyclodextrin are most commonly used experimentally to deplete cells of cholesterol. For example, exposure of cells to 5–10 mM  $\beta$ -cyclodextrins for  $>2$  h removes 80–90% of cellular cholesterol (171). In addition, complexation of  $\beta$ -cyclodextrin with cholesterol is used to deliver cholesterol to cells (172).

Accumulating evidence indicates that the primary mechanism by which CYCLO mobilizes cholesterol from LEs/Ls of NPC-deficient cells depends on bulk-phase

endocytosis (173) or clathrin-mediated endocytosis (174) of CYCLO into LEs/Ls. Time course experiments have shown that cholesterol removal from LEs/Ls requires the internalization of CYCLO into the cells, rather than extraction of cholesterol from the plasma membrane (173). For example, CYCLO was covalently linked to fluorescent dextran, and trafficking of the fluorescent conjugate was visualized in NPC-deficient cells. Cholesterol was efficiently removed from LEs/Ls and was CYCLO localized to intracellular organelles (173). In addition, LE/L cholesterol was mobilized in *Npc1*<sup>-/-</sup> fibroblasts even by incubation with CYCLO that was preloaded with cholesterol (173). Several studies have shown that cholesterol released from LEs/Ls of NPC-deficient cells by CYCLO reaches the cytosolic compartment and is accessible to the ER. An indication of increased ER cholesterol content is that production of cholesteryl esters (the storage form of cholesterol) via the ER localized enzyme ACAT is stimulated. Indeed, cholesterol esterification by ACAT was increased by up to 14-fold in various *Npc1*<sup>-/-</sup> mice tissues and cells after CYCLO treatment (98, 115, 170, 173, 175), demonstrating that the liberated cholesterol must have been accessible to ACAT on the ER. In cultured cerebellar neurons, astrocytes, and microglia from *Npc1*<sup>-/-</sup> mice, the sequestered cholesterol was mobilized to the ER by low concentrations (0.1–1.0 mM) of CYCLO (115). Another indicator of increased cholesterol availability at the ER is decreased expression/processing of SREBP2, a key transcription factor that regulates cholesterol synthesis and uptake (33). CYCLO decreased expression of mRNAs encoding the LDL receptor and 3-hydroxy-3-methylglutaryl-CoA reductase in *Npc1*<sup>-/-</sup> mice and, correspondingly, reduced the rate of cholesterol synthesis in the brain and peripheral tissues (98, 100, 170). These observations clearly demonstrate that in *Npc1*<sup>-/-</sup> cells CYCLO releases cholesterol stored in LEs/Ls so that the cholesterol is mobilized to the ER.

In order to determine which cell types in the brain are responsive to CYCLO, and to investigate further the mechanism by which CYCLO mobilizes LE/L cholesterol in NPC-deficient brains, primary cultures of *Npc1*<sup>-/-</sup> mouse neurons and astrocytes were exposed to 0.1 to 10 mM CYCLO for 24 h. Not surprisingly, because 5–10 mM CYCLO rapidly depletes cholesterol from the plasma membrane, 10 mM CYCLO was profoundly toxic to neurons and markedly altered astrocyte morphology (115). In contrast, 0.1 and 1.0 mM CYCLO were not obviously toxic to these cells and essentially abolished cholesterol accumulation in LEs/Ls of *Npc1*<sup>-/-</sup> neurons, astrocytes, and microglia. Importantly, however, 0.1 mM CYCLO and 1.0 mM CYCLO exerted opposite effects on cholesterol homeostasis. Consistent with the hypothesis that CYCLO releases cholesterol from LEs/Ls to the ER, 0.1 mM CYCLO reduced cholesterol synthesis in *Npc1*<sup>-/-</sup> astrocytes and neurons and profoundly enhanced cholesterol esterification in astrocytes (115). On the other hand, CYCLO did not stimulate cholesterol esterification in cerebellar neurons from *Npc1*<sup>-/-</sup> mice, probably because ACAT activity and cholesteryl ester content are very low in neurons compared with other cells (115). In marked contrast to 0.1 mM CYCLO,

1.0 mM CYCLO increased cholesterol synthesis and expression of genes involved in cholesterol uptake and synthesis in these cells, while dramatically decreasing cholesterol esterification, suggesting that CYCLO had depleted cholesterol even from the ER. These data strongly support the idea that 0.1 mM CYCLO releases cholesterol trapped in LEs/Ls of astrocytes and neurons, thereby increasing the ER content of cholesterol. In contrast, higher concentrations of CYCLO (1.0 mM and greater) appear to extract cholesterol from the plasma membrane and eventually deplete cholesterol from the ER in addition to removing cholesterol from LEs/Ls. These studies indicate that the concentration of CYCLO that would be beneficial in brains of NPC patients is  $\sim 0.1$  mM, consistent with the finding that in brains of *Npc1*<sup>-/-</sup> mice in which CYCLO reversed the cholesterol transport defect, the concentration of CYCLO was  $\sim 0.1$  mM (170). Thus, in brains, and probably also other tissues, concentrations of CYCLO  $>1$  mM would be expected to deplete cellular cholesterol and impair neuron survival (115). On the other hand, in *Npc1*<sup>+/+</sup> mice and cells that do not sequester cholesterol in LEs/Ls, CYCLO does not alter cholesterol homeostasis (115, 176). Even CYCLO that is preloaded with cholesterol can mobilize cholesterol from LEs/Ls without depleting cholesterol from the plasma membrane, suggesting that cholesterol-loaded CYCLO might be less toxic to the brain than CYCLO not loaded with cholesterol (173).

A model for the action of CYCLO that is consistent with the previous data is that CYCLO is taken up by cells via endocytosis and enters LEs/Ls. Cholesterol transfer within the LEs/Ls is normally mediated by the tandem action of NPC1 and NPC2 in a process that requires a physical association between NPC1 and NPC2 (Fig. 1) (27). However, in the presence of CYCLO, cholesterol stored in LEs/Ls is transported to and across the LE/L limiting membrane, independent of either NPC1 or NPC2, and is subsequently transported to the ER (by an undefined mechanism). Because CYCLO reduces cholesterol storage in LEs/Ls of either NPC1- or NPC2-deficient cells (99, 173), CYCLO can bypass the function of both proteins. In vitro studies have shown that CYCLO markedly accelerates cholesterol transfer between membranes via a collisional mechanism involving direct interaction between CYCLO and membranes (177). The mechanism by which CYCLO releases cholesterol from LEs/Ls was further investigated using two types of  $\beta$ -cyclodextrin (167, 176). Previous studies had shown that CYCLO, at concentrations of  $>1$  mM, removes cholesterol from membranes into the aqueous medium by formation of a 2:1 CYCLO-cholesterol molar complex. In contrast, sulfobutylether- $\beta$ -cyclodextrin forms a 1:1 complex with cholesterol that cannot solubilize cholesterol from membranes. At lower concentrations ( $<1$  mM), however, both CYCLO and sulfobutylether- $\beta$ -cyclodextrin can shuttle cholesterol between membranes (171). To determine whether CYCLO removed cholesterol from LEs/Ls of NPC-deficient cells by solubilization or by a shuttle mechanism, *Npc1*<sup>-/-</sup> mice were injected with CYCLO or sulfobutylether- $\beta$ -cyclodextrin (167, 176). With both types of cyclodextrin, the mobilized cholesterol did not rapidly

(at least within 12 h) enter the plasma, either in lipoproteins or in cholesterol-CYCLO complexes, nor was the released cholesterol excreted into urine (176). Furthermore, both cyclodextrins reduced expression of SREBP-responsive genes to a similar extent. Thus, the transfer of stored cholesterol out of LEs/Ls of *Npc1*<sup>-/-</sup> cells does not depend on the ability of CYCLO to solubilize cholesterol into an intracellular or extracellular aqueous milieu. More likely, CYCLO shuttles cholesterol between luminal vesicles within LEs/Ls and the LE/L limiting membrane, so that the cholesterol reaches the exit site on the limiting membranes of LEs/Ls in the absence of NPC1 and NPC2 (176). In an attempt to increase the efficiency of cholesterol clearance from LEs/Ls, supramolecular complexes of cyclodextrin (polyrotaxanes) were designed in which cyclodextrin was threaded onto a biocompatible, linear polymer that was capped by bulky end groups that were amenable to enzymatic cleavage (178, 179). These complexes are endocytosed by cells, and cyclodextrin is released by enzymatic cleavage of the end caps so that high concentrations of cyclodextrin can be delivered to LEs/Ls, while cyclodextrin's activity on the plasma membrane is avoided. Thus, treatment of NPC2-deficient fibroblasts with CYCLO-polyrotaxane complexes efficiently mobilized cholesterol from LEs/Ls (178, 179). Whether these complexes will also be effective in the CNS remains to be determined.

Several other neurodegenerative disorders, such as Alzheimer's disease and Huntington's disease, are also associated with impaired cholesterol homeostasis in the brain (reviewed in Ref. 180). Alzheimer's disease and NPC disease have some similarities, including alterations in lysosomal function (39, 181), increased deposition of amyloid plaques (182–184), and the presence of neurofibrillary tangles in humans (185) [but not in *Npc1*<sup>-/-</sup> mice (106)]. NPC1 expression is higher in the hippocampus and frontal cortex of Alzheimer's patients than in controls (186). Moreover, genetic overexpression of amyloid precursor protein in *Npc1*<sup>-/-</sup> mice decreased their life span, whereas CYCLO prolonged survival (187). CYCLO reduced cholesterol sequestration in brains of mouse models of Alzheimer's disease and lowered levels of amyloid by reducing  $\beta$ -cleavage of amyloid precursor protein and increasing expression of genes involved in clearance of  $\beta$ -amyloid plaques (188). These observations suggest that CYCLO might be a useful therapeutic agent for treatment of Alzheimer's disease and, perhaps also, of other neurodegenerative disorders in which cholesterol homeostasis is disrupted.

## CONCLUSIONS

Identification of the defective genes in NPC disease led to elucidation of the mechanism by which NPC1 and NPC2 proteins mediate the egress of cholesterol from LEs/Ls. Currently, no effective treatment is available for NPC disease patients, although the remarkable discovery that treatment of *Npc1*<sup>-/-</sup> and *Npc2*<sup>-/-</sup> mice with the cholesterol-sequestering agent CYCLO improves the neurodegeneration

and prolongs life are very encouraging. Clearly, however, because of the low permeability of the blood-brain barrier to CYCLO, administration of CYCLO to the CNS of NPC patients is problematic. Thus, a reasonable protocol might be to administer CYCLO simultaneously to the periphery and also to the brain of NPC patients. Such an approach might treat both the liver disease and the neurodegeneration. An unresolved issue, however, is that CYCLO is ineffective against lung disease in NPC-deficient mice. The most effective, nontoxic concentration of CYCLO in the brain must also be established in NPC patients. Another important requirement for clinical studies with CYCLO in NPC patients is that a reliable and convenient method must be developed for assessment of clinical progression of the disease. The observation that CYCLO treatment of NPC-deficient cats caused a hearing defect raises some concerns about this side effect in humans treated with CYCLO. Although CYCLO is a very promising agent for preventing the neurodegeneration in NPC disease, it is unlikely that CYCLO or any of the other proposed treatments would be able to replace neurons that had already been lost. Thus, one of the greatest challenges for treatment for NPC patients is obtaining an early diagnosis of the disease. 

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