Niemann-Pick Disease Type C: Unraveling the Mechanisms Behind a Lysosomal Storage Disease and Their Connection to Neurodegeneration

Hannah Mummey

1Biochemistry Program, Wellesley College, 21 Wellesley College Road, Wellesley, MA 02481, USA.

Abstract
Niemann-Pick Type C (NPC) is a fatal lysosomal storage disease caused by mutations to the NPC1 and NPC2 genes. NPC is characterized by an intracellular accumulation of cholesterol, however the relationship between this and the resulting neurodegeneration is unclear.

Introduction
Niemann-Pick Disease Type C (NPC) is a rare autosomal recessive disease which is estimated to affect 1 in 120,000 people worldwide, although the true occurrence is likely higher (Vanier and Millat, 2003). The onset of the disease is early and most patients are diagnosed before age five, while only approximately 14% are diagnosed after twenty (Vanier and Millat, 2003). The symptoms a patient experiences are variable, but it has been found that nearly all show a linear progression of disease severity independent of age of onset (Yanjanin et al., 2010). This progression is rapid and most patients die within five to ten years (Vanier and Millat, 2003).

NPC is a lysosomal storage disorder characterized by a large accumulation of cholesterol and other lipids in late endosomes and lysosomes. This is especially pronounced in certain organs and manifests in a broad range of symptoms including hepatosplenomegaly, an enlargement of the spleen and liver, lung disease and neurodegeneration (Yanjanin et al., 2010). Neurodegeneration proceeds at a slower pace than other symptoms and extremely young patients often experience complications related to liver and lung damage (Vanier and Millat, 2003). After age three though, patients are usually identified by neurodegeneration symptoms including motor control problems, dementia and supranuclear vertical gaze palsy - the inability to
lift their eyes (Yanjanin et al., 2010). As the disease progresses these symptoms become even
more severe and significantly impact quality of life. Eventually NPC patients have trouble
swallowing and speaking, experience recurrent seizures and become reliant on a wheelchair
(Yanjanin et al., 2010).

There is currently no cure, nor any FDA approved treatment, for NPC. Considering this
deficiency, as well as the intense progression of the disease, it is obvious how formidable NP
C is. In this paper, I review the incomplete knowledge on the mechanisms of NPC and how they
relate to the neurodegenerative symptoms of the disease. I then apply this knowledge to
discuss potential therapeutics currently in development.

**Genetic and Structural Basis of NPC**

NPC is caused by mutations to one of two genes, termed *NPC1* and *NPC2*. Although both
genes lead to the same spectrum of symptoms and are classified as the same disease, the
majority of NPC2 patients experience neonatal onset and markedly severe early symptoms
(Vanier and Millat, 2003). The mutations causing this are mostly nonsense and frameshift but
are very rare and only account for 5% of affected individuals (Vanier and Millat, 2003).
Subsequently, 95% of NPC patients have one of almost three hundred different *NPC1*
mutations, 71% of which are missense mutations spread throughout most domains of the
proteins (Vanier and Millat, 2003).

Until recently, the structures of NPC1 and NPC2 were unknown and it was only possible to
predict their cellular functions. However, in the past few years crystallography has been able to
ascertain these structures. NPC1 codes for a 1278 amino acid long integral membrane protein
with 13 transmembrane regions (Li et al., 2016). Five of these make up a highly conserved
“Sterol-Sensing Domain” (SSD), which is found in other proteins important for cholesterol
binding and biosynthesis (Li et al., 2016). The SSD contains a cavity capable of holding one
cholesterol molecule which is accessible from both the endosomal lumen and the membrane
bilayer (Li et al., 2016). NPC2 is a 132 amino acid free floating protein found in the lumen of the
endosome which binds to cholesterol with high affinity (Xu et al., 2007). The protein can do so
because it contains a hydrophobic sterol binding pocket which expands to fit tightly around a
broad range of sterols (Xu et al., 2007).
Based on these structures, the following is the most recent model of NPC1 and NPC2 function in relation to cholesterol movement. All cholesterol which ends up in the endosome is taken up from extracellular space as part of a low density lipoprotein (LDL) (Wojtanik and Liscum, 2003). LDLs are broken down in the late endosome, after which Lysosomal Acid Lipase (LAL) is responsible for processing the released cholesterol esters into cholesterol (Liu et al., 2009). Then unesterified cholesterol binds NPC2 which docks to the N-terminal domain (NTD) of NPC1 (Figure 1a). Cholesterol is then transferred between NPC2 and the NTD of NPC1 which wraps around and passes the cholesterol molecule to the SSD via the lumen side of the pocket (Figure 1b,c). Upon reaching this pocket cholesterol is then able to exit into the lysosomal lipid bilayer via the lateral opening of the SSD (Figure 1d). It is important to note that from here cholesterol would need to be flipped into the cytosolic leaflet and transferred to the cytosol and it is unknown if NPC1 is involved in these processes. Regardless, without either NPC1 or NPC2, cholesterol remains trapped in the endosomal lumen.

Figure 1: Function of NPC1 and NPC2 in Cholesterol Transport from the Endosomal Lumen. The crystal structures of NPC1 (blue, green, purple and orange subunits) and NPC2 (yellow) and their proposed interaction with cholesterol are shown. a) and b) illustrate the transfer of cholesterol between NPC2 and the NTD of NPC1. c) shows the transfer from the NTD to the SSD in NPC1. The cytosol (C) and endosomal lumen (L) as shown in c) are consistent across all panels. d) shows how cholesterol can then diffuse out into the luminal leaflet of the membrane. In each image the cholesterol molecule is circled in red. Figure adapted from Li et al. (2016).
Cellular Importance of NPC1 and NPC2

Even before the structures and mechanisms of NPC1 and NPC2 were known, it was possible to study the effects of their absence on cholesterol and the endosomal pathway. The natural life cycle of an endosome involves tubulin facilitated movement towards the nucleus accompanied by gradual development into a lysosome. Before the transition to a lysosome, NPC1 containing vesicles bud off from late endosomes and deliver cholesterol to perinuclear compartments, like the Endoplasmic Reticulum (ER) (Wojtanik and Liscum, 2003). However, in NPC cells ER cholesterol levels do not rise in response to increased extracellular LDL as they should (Frolov et al., 2003). Instead if NPC1 or NPC2 is dysfunctional cholesterol remains in late endosomes and travels directly to the lysosome where it accumulates (Wojtanik and Liscum, 2003). This is in accordance with the proposed role of the proteins in export of cholesterol from the lumen.

By preventing the extraction of cholesterol from late endosomes, NPC mutations also impair the ability for cholesterol to be esterified, which is important for maintaining sterol homeostasis (Radhakrishnan et al., 2008). Cholesterol is esterified in the ER however when ER cholesterol content falls to around 5% the Sterol Regulatory Element Binding Protein (SREBP) is activated (Radhakrishnan et al., 2008). This is a transcription factor promoting genes important for cholesterol synthesis (Radhakrishnan et al., 2008). Thus, when cholesterol does not arrive at the ER more is signaled to be formed, furthering the disruption of homeostasis.

The generation of oxysterols is also important for maintaining cholesterol homeostasis and is impaired in NPC. Oxysterols serve as a negative feedback mechanism for cholesterol uptake by inhibiting LDL receptor activity (Frolov et al., 2003). Removal of cholesterol from the endosome is a prerequisite to oxysterol formation and NPC cells experience a delay in the regulation of LDL receptor (Frolov et al., 2003). Accordingly, when supplemented with oxysterols this was fixed and NPC cells were able to regain cholesterol homeostasis (Frolov et al., 2003).

Role of Membrane Microdomains

Although the disease associated mutations impair proper cholesterol localization, there is a growing pool of evidence that the underlying cause of many NPC symptoms is the accumulation of other lipids. The primary hypothesis is that the creation of enduring lipid rafts facilitates this. Accumulation of cholesterol in late endosomes also recruits glycosphingolipids (GSLs) and together these make up lipid rafts, or membrane microdomains. Normally membrane
microdomains are present in early endosomes but disperse as the endosome develops (Vruchte et al., 2004). However, in NPC cells it has been found that the high levels of cholesterol allow these microdomains to persist and they continue to be found in late endosomes (Vruchte et al., 2004).

Because of this, NPC is also characterized by an accumulation of glycosphingolipids in the endosomal pathway. Treatment of the NPC mouse model with N-butyldeoxynojirimycin (NB-DNJ), an inhibitor of GSL synthesis, reverses several cellular disease phenotypes without influencing cholesterol concentrations (Vruchte et al., 2004). This reveals that GSLs also contribute significantly to the disruption of cellular function in NPC, likely due to their build up in late endosomal microdomains.

Many proteins involved in cellular transport, including Annexin 2 and 6, and Rab 7, associate with these microdomains and so the presence of abnormal lipid rafts also promotes mis-localization and trafficking problems (Vruchte et al., 2004). Although endosomes move towards the nucleus, late endosomes are capable of bidirectional movement facilitated by kinesins and dynein (Lebrand et al., 2002). Rab7 interacts with dynein and promotes minus-end movement towards the nucleus (Lebrand et al., 2002). The creation of microdomains prevents the extraction of Rab7 and this results in endosomes becoming stuck in the perinuclear region (Lebrand et al., 2002). The inability of endosomes to move about the cell could further impair trafficking for all molecules, not just lipids.

It has also been proposed that membrane rafts are not as prevalent in NPC as suspected. In one study, the lysosomes of NPC cells experienced a 2-3 fold increase in size but the relative concentration of lipid raft domains did not change (Sobo et al., 2007). Instead the late endosome had reduced intra-organellar trafficking. Complex sorting processes occur in the endosome, including the formation of intraluminal vesicles. In NPC cells the ability of intraluminal vesicles to fuse back with the lysosome is altered, resulting in a decreased interaction between the two membranes (Sobo et al., 2007). This interferes with the sorting of certain proteins in the endosome and consequently their transport throughout the cell. Although conflicting data exist, there is a clear pattern that intracellular trafficking is impaired in NPC which can lead to a variety of life-threatening symptoms.
Connection to Neurodegeneration

Many patients, especially younger ones, die from complications due to symptoms in the visceral organs like the lungs and the liver. However, the relation between cholesterol accumulation and these symptoms is reasonably straightforward. NPC patients also experience life threatening neurodegeneration, with the most affected regions being the cerebellum and brainstem (Liao et al., 2007). The cerebellum is responsible for coordinating movement and accordingly most patients display impaired motor control (Yanjanin et al., 2010). The brain contains approximately 25% of the body’s cholesterol, so it makes sense that dysregulation of cholesterol trafficking would greatly affect it. However, the mechanisms tying cholesterol buildup to neurodegeneration in NPC are still poorly understood.

In NPC1 knockout mice cholesterol was observed to accumulate to stainable levels even before most symptoms of neurodegeneration appeared (Reid et al., 2004). This early accumulation is especially notable in the dendrites of Purkinje neurons which suggests it localizes here in NPC (Reid et al., 2004). A lack of proper intracellular dispersal could lead to cholesterol deprivation in other parts of the neuron, like synapses. Cerebellum levels of neurotransmitters are abnormal in NPC mice (Hawes et al., 2010), which suggests that cholesterol may have a role in neurotransmitter secretion, but this has yet to be confirmed. Signaling is important, not only to convey information necessary for normal body function, but also for the neuronal life cycle. Neurons which cease to send or receive signals are often targeted for degradation and so this impairment of signaling may help explain why neurodegeneration occurs.

A few other possible causes of neuronal death are unnecessary apoptosis and the activation of the neuroimmune system; however, there is evidence that neither is a causative factor. NPC knockout mice were found to have high levels of inactive GSK-3beta, which is indicative of an overactive PI3K pathway, an anti-apoptotic pathway (Bi et al., 2005). In other mouse studies the release and localization of complement was not correlated with neuronal death (Lopez et al., 2012). The complement system stimulates and enhances the ability of microglia, phagocytic cells in the neuroimmune system, but interference with the pathway did not affect neurodegeneration (Lopez et al., 2012). This suggests that the neurological inflammation response may only be a result of cell death. Similarly, NPC cells were found to have elevated secretion of IL-6, a cytokine which activates glial cells (Suzuki et al., 2007). Upon activation glial cells can contribute to Purkinje degeneration, however this was also found to not be initially causal (Suzuki et al., 2007).
**Relationship Between Neurodegeneration and Autophagy**

Another potential mechanism of neurodegeneration is based around the cellular process of autophagy. Autophagy is the pathway through which internal membranes form around macromolecules in the cytosol to create autophagosomes which then fuse with lysosomes. This process is involved in programmed cell death II (Liao et al., 2007) but it does not necessarily lead to it. When properly controlled, autophagy is also important for recycling organelles and eliminating faulty or superfluous proteins. Most neurons have constant levels of autophagy and it has recently been postulated to be important for normal neuronal growth and development (Nikoletopoulou et al., 2015). Suppression of autophagy has been linked with neuronal death (Hara et al., 2006) and thus connections are being drawn between many neurodegenerative diseases like NPC and dis-regulation of autophagy (Nikoletopoulou et al., 2015).

Many markers of autophagy are elevated in NPC cells. Basal autophagy is partially facilitated by the protein Beclin-1, part of a PI3K complex which helps to form autophagosomes (Pacheco et al., 2007). In NPC cells expression of Beclin-1 is increased by accumulation of cholesterol, however knockdown of Beclin-1 was only able to decrease autophagy by 20% (Pacheco et al., 2007). The overexpression of Beclin-1 may not directly lead to autophagy but instead prime cells for it, allowing other unknown factors to easily trigger it. Raised levels of cholesterol in NPC cells are also linked to an increase in LC3-II marked granular structures (Ishibashi et al., 2009). LC3-II, a microtubule associated protein, binds to the membrane of autophagosomes and is a reliable indicator of autophagy as it is also degraded upon fusion with the lysosome (Ishibashi et al., 2009). The increase of granular structures corresponds to higher levels of initiation of autophagy but could also indicate an accumulation of autophagosomes.

The elevated amount of autophagosomes in NPC cells also indicates that the pathway flux is disrupted (Ishibashi et al., 2009). If initiation of autophagy is increased, but flux does not match this, there will be an accumulation of autophagosomes. This disrupts pathway homeostasis and leads to cellular stress by preventing the formation of new autophagosomes. This could also result in a failure to remove damaged organelles and proteins which might be fatal to neurons because they rely on a fast turnover of macromolecules (Hara et al., 2006). The buildup of autophagosomes could also interfere with cellular trafficking, as large amounts of membrane are sequestered. Although the disruption of autophagy homeostasis appears to have promising ties to neurodegeneration, there is still much research that needs to be done to understand the complete causes of it.
Potential Therapies for NPC

Because the relationship between lysosomal cholesterol accumulation and NPC symptoms is still unclear it is hard to find effective therapies. Developing treatments which can reach organ systems on both sides of the blood brain barrier is an additional challenge. There is a precedent of treatment by bone marrow transplant in lysosomal storage diseases (Hsu et al., 1999). Unfortunately, when NPC affected individuals were tested with this treatment only visceral disease markers were improved, and neurodegeneration continued (Hsu et al., 1999). Additionally, treatment by imatinib was able to reduce Purkinje cell death, however mouse model lifespan only increased by 12% (Alvarez et al., 2008). This affirms that both neurological and visceral symptoms need to be addressed to improve patient health and lifespan.

Although there is no treatment approved directly for NPC, miglustat, a small molecule drug for Gaucher’s disease, can have beneficial effects (Patterson et al., 2007). As discussed earlier, although NPC is the result of mutations to cholesterol associated proteins, GSLs play an important role in the cellular mechanism of disease. Miglustat is an iminosugar which inhibits the synthesis of glucosylceramide, the precursor to all GSLs, and lowers cellular concentrations of them (Patterson et al., 2007). Treatment with miglustat was shown to correct abnormal lipid trafficking in NPC B cells, while reduction of cholesterol had no effect (Lachmann et al., 2004). This supports the idea that the accumulation of glycosphingolipids in the lysosome may be the true cause of NPC symptoms, rather than cholesterol. Although miglustat is used by some NPC patients, it can only be prescribed “off-label” which cannot be covered by all insurance plans and thus it is not an accessible drug.

Another promising treatment currently in development is the use of the cyclodextrin 2-Hydroxylpropyl-beta-cyclodextrin (HPBCD). Cyclodextrins are a class of molecules which consist of cyclical rings of 6 to 8 glucopyranose units. Because of this ring structure, cyclodextrins are mainly hydrophilic but contain a lipophilic hydrophobic core which can solubilize hydrophobic lipids (Liu et al., 2009). Treatment of the NPC mouse model with a single dose of HPBCD led to an increase in the concentration of cholesterol esters and a suppression of overall cholesterol synthesis (Liu et al., 2009). Both result from actions of free cytosolic cholesterol or ER associated cholesterol and thus are indirect measures (Radhakrishnan et al., 2008). However, they serve as strong indicators that HPBCD reduces lysosomal cholesterol levels. Concurrently, treated mice had an increased lifespan, improved liver function and reduced levels of neurodegeneration and all improvements lasted for multiple weeks after the
dose (Liu et al., 2009). Although these results are promising, 2HPBCD is unable to cross the blood brain barrier easily and direct delivery to the central nervous system is necessary to fully combat neurodegeneration (Aqul et al., 2011).

While the benefits of HPBCD treatment are clear, the cellular mechanisms through which they are accomplished are not. Due to their unique structure, cyclodextrin molecules are able to bind and extract sterols from membranes in vitro (Aqul et al., 2011). Many authors attribute the release of cholesterol from NPC lysosomes to this mechanism. But it is unknown if a cyclodextrin molecule would be able to cross the plasma membrane and lysosomal membrane to access sequestered cholesterol. Another hypothesized mechanism connects HPBCD treatment with lysosomal exocytosis (Chen et al., 2010). However, this would deposit all accumulated cholesterol extracellularly rather than into the cytosol and how this connects to the observed increase in intracellular cholesterol levels is not clear (Chen et al., 2010).

It should be noted that if lysosomal cholesterol levels are extremely high, just one dose of HPBCD is not enough to relieve them (Chen et al., 2010). HPBCD exposure for over 20 consecutive hours was necessary to significantly decrease cholesterol levels of NPC cells (Chen et al., 2010). Regardless, because NPC and related lysosomal storage diseases vary in their rate of progression, HPBCD could still have important therapeutic use if diagnosis is early. This is like miglustat which can prevent GSL synthesis, but not influence cholesterol levels (Patterson et al., 2007).

**Outlook**

Although miglustat and cyclodextrin seem like promising therapies, they both have yet to gain FDA approval. In 2010 the application for an expansion of miglustat's use to treat NPC was denied and more preclinical and clinical data was requested (Ltd., 2010). Additionally, it was announced in late 2018 that a trial of VTS-270 (HPBCD) did not find significant differences between the treated and placebo groups (Wadman, 2018). More unexpected however is that both groups experienced a slower disease progression, perhaps indicating a problem in the study design, rather than the drug (Wadman, 2018). These stumbles have only served to delay the existence of a fully accessible treatment drug for NPC and leave many patients without effective therapy for a deadly disease.
Much remains to be discovered about the roles and mechanisms of NPC1 and NPC2. For example, NPC1 has been identified as instrumental for infection by and pathogenesis of the Ebola and Marburg viruses (Herbert et al., 2015). This effect occurs regardless of cellular cholesterol transport levels (Herbert et al., 2015), and thus must be reliant upon an additional unknown function of NPC1. Surprising findings like this illustrate the lack of a complete understanding of the protein’s function and cellular mechanisms. Both miglustat and HPBCD are reactionary treatments which only slow disease progression and rely on early identification. If we better understand how NPC1 and NPC2 function and the effects of lysosomal lipid accumulation, we may be able to develop treatments which can supplement this. The ability to reverse disease symptoms rather than just treat them would be revolutionary for the care of NPC patients.
References


Essential for Ebolavirus Replication and Pathogenesis In Vivo. mBio 6, e00565-00515.


