Journal of Neurochemistry

JNC

JOURNAL OF NEUROCHEMISTRY | 2019 | 153 | 674–692

doi: 10.1111/jnc.14895

REVIEW

# Niemann–Pick type C disease: cellular pathology and pharmacotherapy

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**Abstract**

Niemann–Pick type C disease (NPCD) was first described in 1914 and affects approximately 1 in 150 000 live births. It is characterized clinically by diverse symptoms affecting liver, spleen, motor control, and brain; premature death invariably results. Its molecular origins were traced, as late as 1997, to a protein of late endosomes and lysosomes which was named NPC1. Mutation or absence of this protein leads to accumulation of cholesterol in these organelles. In this review, we focus on the intracellular events that drive the pathology of this disease. We first introduce endocytosis, a much-studied area of dysfunction in NPCD cells, and survey the various ways in which this process malfunctions. We briefly consider autophagy before attempting to map the more complex pathways by which lysosomal cholesterol storage leads to protein misregulation, mitochondrial dysfunction, and cell death. We then briefly introduce the metabolic pathways of sphingolipids (as these emerge as key species for treatment) and critically examine the various treatment approaches that have been attempted to date.

**Keywords:** cholesterol, endocytosis, glucosylceramide, GBA2, lysosome, Niemann-Pick.

*J. Neurochem.* (2020) **153**, 674–692.

## NPC1

Despite years of research the functions of NPC1 still resist simple definition. It has been proposed as an exporter of lipophilic molecules (Davies *et al.* 2000a) and amines, (Kaufmann and Krise 2008) and to bind sphingolipids (Malathi *et al.* 2004) and mycolic acids (Fineran *et al.* 2017). However, the emphasis has been on this protein as a cholesterol exporter following the recognition that it possess a sterol sensing domain (SSD) (Carstea et al. 1997). Mutations in the SSD were found to impede cholesterol export from the lysosome (Millard *et al.* 2005) and to reduce labelling of the protein by a fluorescent cholesterol analogue (Ohgami *et al.* 2004). The developing view of the pathogenesis of NPCD was complicated somewhat by the discovery that a second lysosomal protein, termed NPC2, was also involved in the disease (Naureckiene *et al.* 2000). However, this molecule was swiftly established as capable of binding cholesterol (Ko *et al.* 2003; Friedland *et al.* 2003) and subsequent studies confirmed this (Xu *et al.* 2007; Infante *et al.* 2008a). The idea that NPC1 and −2 act together was put forward (Subramanian and Balch 2008; Infante *et al.* 2008b) and has been confirmed and refined by later work. Thus within the lysosome NPC2 collects cholesterol (Berzina *et al.* 2018) and conveys it to the N-terminal domain (NTD) of NPC1 in the limiting membrane. The transfer of cholesterol from NPC2 to NPC1(NTD) has not yet been observed in live cells but has been studied in model systems (Infante *et al.* 2008b; Wang *et al.* 2010) and computationally (Hodošček and Elghobashi-Meinhardt 2018). It is believed that NPC1 then transfers cholesterol from the NTD to the SSD; the mechanism of this transfer is unknown but recent work identifying a hydrophobic tunnel in a yeast orthologue may offer significant insights (Winkler *et al.* 2019) and is consistent with an *in silico* study based on a low-resolution crystal structure (Elghobashi-Meinhardt 2019). Binding of cholesterol in the SSD has been inferred from mutational studies (Ohgami *et al.* 2004; Millard *et al.* 2005) but to date binding sites have been identified with precision only by computational means (Li *et al.* 2016; Elghobashi-Meinhardt 2019; Wheeler *et al.* 2019b). These studies produce varying results and agree only that NPC1 possesses a cholesterol-binding site aligned with the luminal leaflet of the lysosomal limiting membrane; it thus remains an open question whether or not NPC1 is sufficient to complete the export of cholesterol or whether other entities are involved. It has been proposed (Li *et al.* 2016) that cholesterol diffuses away

Received July 1, 2019; revised manuscript received September 10, 2019; accepted September 15, 2019.

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*Abbreviations used*: SSD, sterol sensing domain; NTD, N-terminal domain; LSDs, lysosomal storage disorders; LDL, low density lipoprotein; CLICs, clathrin-independent carriers; AnxA2, annexin A2; LBPA, lysobisphosphatidic acid.



 

from NPC1 into the membrane where it would be expected to flip rapidly (Steck and Lange 2012) to become aligned with the cytosolic face from where it could be collected by a carrier protein. Its distribution may be regulated by C24-sphingomyelin (Courtney *et al.* 2018) which in turn can be flipped by ABCA1 (Choi *et al.* 2003; Quazi and Molday 2013) which localizes to endosomes (Quazi and Molday 2013). Whether glucosylceramide on the cytosolic face of the membrane (to be discussed later) can perform the same function as sphingomyelin has not yet been investigated.

While the mechanism by which NPC1 completes cholesterol export has not been fully elucidated, beyond controversy (e.g., Carstea *et al.* 1997) is that mutant NPC1 or −2 lead to accumulation (often termed storage) of cholesterol in late endosomes and lysosomes of NPCD patients. This condition thus constitutes a member of the class of lysosomal storage disorders (LSDs) but is unusual in that category as most such diseases derive from failure of catabolism rather than from failure of export. Cholesterol in the lysosome is mostly derived from low density lipoprotein (LDL) via the process of endocytosis.

## Endocytosis

Endocytosis is a constitutive process by which cells internalized nutrients and refresh their outer membranes. There are at least three varieties known to modern biology. The best studied depends on the protein clathrin which accumulates in regions of the plasma membrane and forms pits. These pits contain certain receptors such as that for LDL and the iron-binding protein transferrin. Clathrin induces a curvature in the membrane that eventually becomes so pronounced that the pit is almost separated from the membrane; the protein dynamin completes the separation and an approximately spherical, clathrin-coated vesicle forms. The coat is soon shed and the resulting vesicle proceeds to carry its cargo into the cell. A less well understood mechanism uses the protein caveolin to induce membrane curvature and form structures known as caveolae; the insulin receptor is internalized in this way (Gustavsson *et al.* 1999). Once a caveola has been pinched off it too forms a vesicle. Similarly, the protein galectin-3 can form structurally distinct vesicles known as clathrin-independent carriers (CLICs) (Lakshminarayan *et al.* 2014). This process depends on glycosphingolipids, (Lakshminarayan *et al.* 2014) a class of compound we will meet much more extensively later. Once the vesicles are generated by whichever mechanism they eventually fuse with bodies known as early endosomes. From here some material is recycled to the cell surface while other is progressed along the endocytic pathway to late endosomes. The regulation of this process is not fully understood but involves both rab proteins and phosphoinositide lipids (Shen *et al.* 2011; Li *et al.* 2013). Late endosomes are also known as multi-vesicular bodies (MVBs) as they feature intra-luminal membranes which is where the original endocytic cargo resides.

Further processing requires the late endosome to fuse with a lysosome. This in turn necessitates them being close in space meaning both organelles must be mobile. The small GTPase rab7 attached to the membrane of each organelle contacts motor proteins that attach to the cytoskeleton (Lebrand *et al.* 2002; Chen *et al.* 2008; Rocha *et al.* 2009) and allow the requisite movement. (As rab7 is present on both late endosomes and lysosomes it is not possible to distinguish the two sets of organelles precisely so the term 'late endolysosome' (LEL) will be used here to include both). Merging of endocytic vesicles may also require annexin A2 (AnxA2), (Mayran *et al.* 2003; Morel and Gruenberg 2009) possibly through its ability to mediate membrane fusion events (Grill *et al.* 2018). The definitive requirements are the initial formation of a tether between rab7 and a cognate effector, probably the HOPS complex, (Luzio *et al.* 2007) release of calcium ions (Pryor *et al.* 2000) and finally formation of a SNARE complex from proteins on the surface of each partner. For endocytosis these SNAREs are syntaxin 7 (Stx7) and VAMP8 (Ward *et al.* 2000; Pryor *et al.* 2004).

Once the hybrid organelle has formed from an endosome and a lysosome catabolism of the contents is performed by an array of enzymes; the pH optima of many of these enzymes are low (Xiong and Zhu 2016) thus an acidic environment is necessary. Acidity is achieved by the activity of the vacuolar ATPase pump (vATPase) (Forgac 2007) inhibition of which is reported by some studies to lead to widespread failure of endocytosis (van Weert *et al.* 1995; Baravalle *et al.* 2005). Metabolites generated in the LEL are exported by a variety of proteins including NPC1, the lysosome reforms while the rab and SNARE proteins are removed from the membrane to be reused.

In addition to the failure to export cholesterol from the LEL a variety of experiments have detected multiple transport defects in Niemann-Pick cells (Neufeld *et al.* 1999; Liscum 2000; Choudhury *et al.* 2002; Choudhury *et al.* 2004; Pipalia *et al.* 2007; Kaufmann *et al.* 2009; Tharkeshwar *et al.* 2017) including a slowing of the entire endocytic process (Choudhury *et al.* 2004; Tharkeshwar *et al.* 2017). How can an accumulation of a single metabolite have such wide-ranging effects? As we have already seen endocytosis relies on an extensive, complex set of protein machines to function. There is a growing appreciation of the interaction of lipids, especially cholesterol (e.g., Epand 2006; Levitan *et al.* 2014), with proteins and that this interaction has functional consequences. Could the origins of the endocytic defect in NPCD result from protein dysfunction induced by cholesterol accumulation?

Successive investigations have indeed found it to be so. Thus under conditions of high cholesterol content such as pertain in NPCD the endolysosomal protein ORP1L undergoes conformational change (Rocha *et al.* 2009; Vihervaara *et al.* 2011). Through a complex of proteins, including rab7, this associates the motor protein dynein with the organelle. Dynein is responsible for movement to the minus-end of

microtubules, which is to say toward the cell interior. In NPCD the endolysosomal cholesterol content is permanently high so dynein remains associated and the organelles become immobilized (Zhang *et al.* 2001; Lebrand *et al.* 2002; Chen *et al.* 2008; Takahashi and Kobayashi 2009; Rocha *et al.* 2009) away from the cell periphery (Ko *et al.* 2001; Lebrand *et al.* 2002; Rocha *et al.* 2009) and therefore unable to fuse with early endosomes This mechanism also keeps them away from the ER. This is important as the ER is believed to be replenish (Gerasimenko *et al.* 1998) endolysosomal calcium stores which have been used up in vesicle fusion but are needed if the lysosome is to fuse with and therefore process the next wave of incoming nutrients. Indeed lysosomal calcium was found to be low in NPCD cells (Lloyd-Evans *et al.* 2008; Xu *et al.* 2012; Visentin *et al.* 2013) while blocking the $IP_3$ receptor led to a proliferation of endocytic vesicles, (Garrity *et al.* 2016) a characteristic of lysosomal storage diseases.

Cholesterol accumulation also makes a more direct contribution the endocytic defect. Annexins A2 (Harder *et al.* 1997; Mayran *et al.* 2003) and A6 (de Diego *et al.* 2002; Domon *et al.* 2010) both associate with cholesterol-rich areas of membrane and so in NPCD mislocalize to late, rather than early, endosomes; (Domon *et al.* 2011; Te Vruchte et al. 2004) this defect is recapitulated in cells treated with a small molecule blocker of NPC1 (Mayran *et al.* 2003). While the function(s) of annexins in endocytosis have only been partially elucidated (Mayran *et al.* 2003; Morel and Gruenberg 2009; Grill *et al.* 2018) their tendency to associate with cholesterol-rich membranes renders likely some role in the NPCD endocytic defect. Other proteins found in membrane subfractions with high cholesterol content include rab7, (Yu *et al.* 2007) syntaxin7 (Yu *et al.* 2007; Enrich *et al.* 2015) and VAMP8 (Gu *et al.* 2012). As shown above the successful continuation of the endocytic cycle requires their removal from membranes for re-use. This removal is retarded for all these proteins when they are present in cholesterol-rich membranes, (Lebrand *et al.* 2002; Takahashi and Kobayashi 2009; Fraldi *et al.* 2010) such as those present in NPCD endolysosomes, and so endocytosis as a whole is slowed down (Lebrand *et al.* 2002; Takahashi and Kobayashi 2009). (A similar defect affects NPCD synapses (Xu *et al.* 2010b)). Consistently this transport defect can be rescued by overexpression of rab7 (Choudhury *et al.* 2002). (Experiments involving rab7 should be interpreted with caution as this protein has a plethora of functions, (Guerra and Bucci 2016) many of which such as cathepsin D maturation, (Zhang *et al.* 2009) autophagy (Gutierrez *et al.* 2004; Ganley *et al.* 2011) and lysosome-mitochondrion contacts (Wong *et al.* 2018) are likely to be relevant to NPCD. Controlling adequately for all these factors is necessarily challenging.)

Thus, numerous endolysosomal proteins are likely to be affected by high cholesterol content – we have recently contributed an *in silico* modelling study exploring the molecular details of these interactions (Wheeler *et al.* 2019b). Another key protein is the vATPase proton pump through which these organelles acquire the requisite acidic pH. The pH of lysosomes in NPCD cells has been reported as elevated by ourselves (Wheeler *et al.* 2019a) as well as other workers (Tharkeshwar *et al.* 2017; Chakraborty *et al.* 2017) although other studies find it normal (Bach *et al.* 1999; Lloyd-Evans *et al.* 2008; Elrick *et al.* 2012). (We propose that these differences are because of heterogeneity of lysosomal vesicles. Although a subset of endocytic vesicles acidify correctly in NPCD another population is absent (Leung *et al.* 2018); reported differences in endolysosomal pH may therefore be because of different methods measuring dissimilar lysosomal populations.) Acidification will generate an electrical potential (Gerasimenko *et al.* 1998; Ishida *et al.* 2013; Cang *et al.* 2015) and there is debate whether ion fluxes mediated by other ion channels in the endolysosomal membrane are necessary to balance this. The debate has been wide-ranging (Koivusalo *et al.* 2011; DiCiccio and Steinberg 2011; Mindell 2012) and will not be entered here, although the association of calcium, (Sun 2000) chloride (Kasper *et al.* 2005; Poët *et al.* 2006; Pressey *et al.* 2010) and potassium (Lill *et al.* 2015; Jinn *et al.* 2017) channels with neurodegeneration may well be significant. Indeed even the absence of PIKfyve, the enzyme responsible for synthesizing the ligand for some calcium channels, results in neurodegenerative disease (Zolov *et al.* 2012).

### Autophagy

Autophagy is the process by which cells degrade worn out organelles and toxic proteins. It begins with the formation of a double-membraned organelle termed the phagophore which seals to become an autophagosome (Xie *et al.* 2008). This fuses with a lysosome to become an autolysosome wherein the necessary degradation occurs and metabolites are exported.

Given the resemblance of this process to endocytosis we might expect similar requirements to pertain, particularly to the organelle fusion step. Indeed correct acidification, (Kawai *et al.* 2007; Koga *et al.* 2010; Aldrich *et al.* 2015) rab7, (Gutierrez *et al.* 2004; Ganley *et al.* 2011; Wijdeven *et al.* 2016) $Ca^{2+}$ efflux through the TRPML1 channel, (Zhang *et al.* 2016) Stx17 (Itakura *et al.* 2012) (though not −7 (Furuta *et al.* 2010)) and VAMP7 (Fader *et al.* 2009) or −8 (Furuta *et al.* 2010) have all been reported as necessary. The additional dependence on the correct proportion of membrane cholesterol (Koga *et al.* 2010; Wijdeven *et al.* 2016) raises the expectation that autophagy will be slowed in NPCD, just as endocytosis is, and that this deficiency may have similar molecular origins. Such an error would be expected to appear as a proliferation of autophagosomes (visualized as positive for marker protein LC3-II) as they fail to fuse with lysosomes. This has indeed been repeatedly reported in NPCD (e.g., Ko *et al.* 2005; Pacheco *et al.* 2007; Sarkar *et al.* 2013)).

Nonetheless alternative accounts, only partially consistent, are available. Work in NPC neurones (derived from embryonic stem cells, ESCs) found the autophagic pathway not stalled but rather over-active, (Ordonez *et al.* 2012) a result echoed in NPC fibroblasts (Pacheco *et al.* 2007; Elrick *et al.* 2012). On this model the proliferation of autophagic vesicles (e.g., Pacheco *et al.* 2007; Sarkar *et al.* 2013) derives from reduced clearance of autolysosomes and not from impaired fusion of their precursors. In turn clearance may be slowed by reduced activity of lysosomal proteases the cathepsins which was attributed, after elimination of more conventional causes, to direct enzyme inhibition by stored lipids (Elrick *et al.* 2012).

## From cholesterol storage to secondary lipid accumulation

In common with other lysosomal storage disorders various lipids accumulate in NPCD including di- and tri-acylglycerols (Tharkeshwar *et al.* 2017), phosphoinositides (Tharkeshwar *et al.* 2017), sphingosine (Te Vruchte *et al.* 2004), sphingomyelin (Vanier 1983; Harzer *et al.* 2003; Tharkeshwar *et al.* 2017), GlcCer (Harzer *et al.* 2003) and gangliosides (Te Vruchte et al. 2004; Zhou et al. 2011). This results from the interplay of at least three factors: decreased lysosomal acid lipase activity because of increased pH (van der Poel *et al.* 2011), favourable physico-chemical interactions between lipids (Picas et al. 2016; Engberg *et al.* 2016) and reduced enzyme activity because of stored lipids (Sandhoff and Sandhoff 2018). Sometimes the result of this interplay is surprising. Thus although NPCD cells accumulate sphingomyelin, mice where this lipid's lysosomal hydrolase has been knocked out (to simulate Niemann-Pick type A disease) do not accumulate cholesterol at the whole cell level (Scandroglio *et al.* 2008; Galvan *et al.* 2008; Camoletto *et al.* 2009). So it becomes apparent that subcellular lipidomics is necessary to quantitate lipid accumulation (see Tharkeshwar *et al.* 2017 for NPCD); indeed the anisotropic distribution of lipids, and the potentially key role of minor lipid populations (Devlin *et al.* 2010; Wheeler *et al.* 2019a), suggest that suborganellar lipidomics (Kobayashi *et al.* 2002) may be necessary for an understanding of this disease. In particular, the question of whether cholesterol accumulates at intralysosomal membranes (as shown in Fig. 1) or the limiting membrane or both remains unanswered. Performing these experiments in cells from a variety

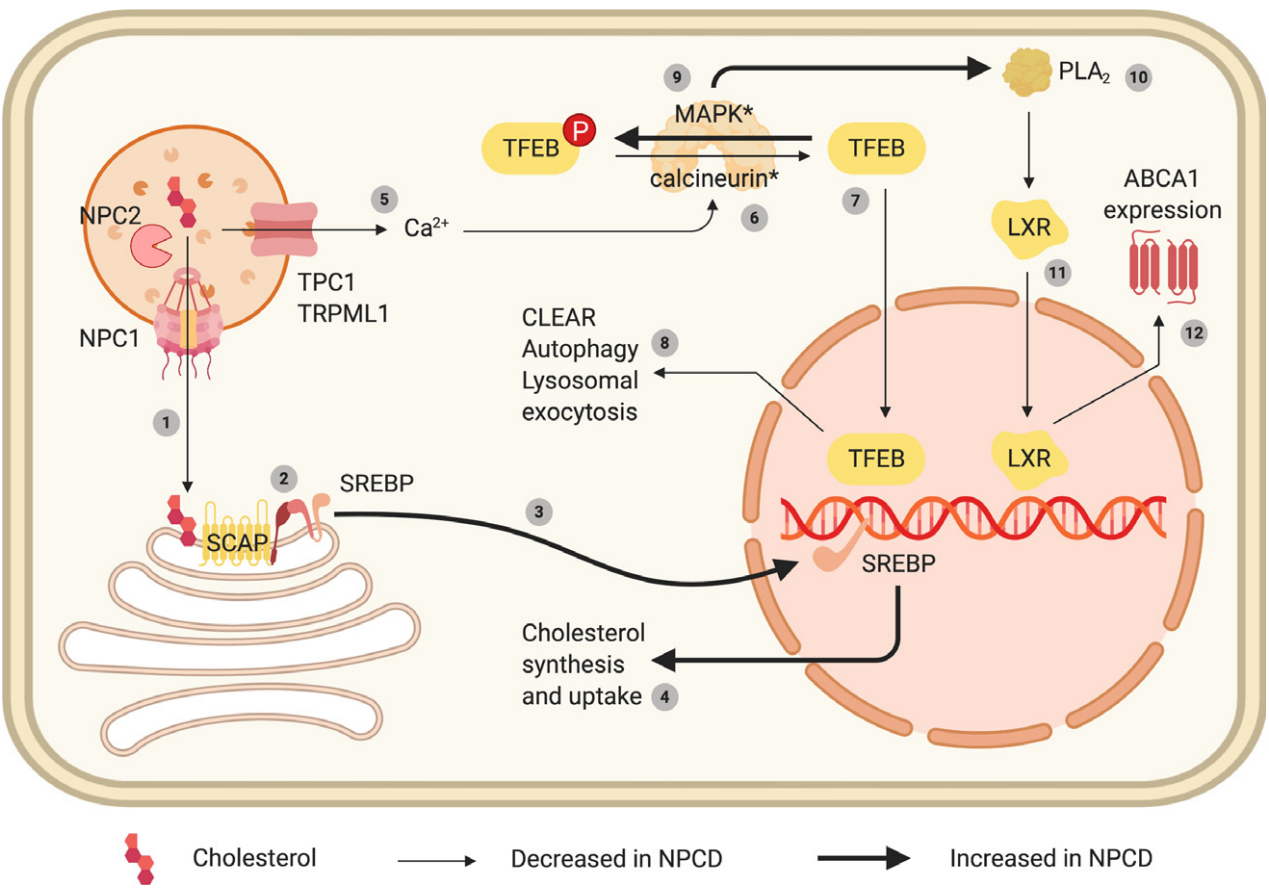


**Fig. 1** From cholesterol storage to protein misregulation Cholesterol storage in NPCD sets off chains of events that lead ultimately to the overexpression of certain proteins and the under-expression of others. For details see text.

of LSDs may shed light on any common pathological pathways, including the possibility that secondary lipid storage is responsible for deleterious cellular effects, an issue which will be addressed again later.

### From cholesterol storage to protein misregulation

It is becoming increasingly apparent that lysosomes, rather than being merely waste processing units, are fully integrated into the life of the cell (Lie and Nixon 2019). Accordingly, failure of lysosomal metabolite export will have effects that cannot be contained to the organelles directly affected but will impact the whole cell. In the case of cholesterol the onward destination from the lysosome has not been fully elucidated – there seem to be multiple cholesterol transport pathways operating simultaneously (van der Kant and Neefjes 2014; Raiborg *et al.* 2015; Pfisterer *et al.* 2016) and other proteins seem to have roles that have yet to be discovered (Bishop and Woodman 2000; Du *et al.* 2013). However, it is generally agreed that NPCD results in a cholesterol deficit at both the ER (Frolov *et al.* 2003; Du *et al.* 2011) and the Golgi (Garver *et al.* 2002a) with consequences for protein regulation (Fig. 1 where the suffix * denotes a protein in its activated state).

Reduced levels of cholesterol at the Golgi (Garver *et al.* 2002a) (Fig. 1, ❶) affect the sterol homeostatic protein SREBP and its regulator SCAP. Under normal conditions cholesterol binds to SCAP inducing conformational change and keeping it associated with SREBP (Fig. 1, ❷). Under conditions of low Golgi cholesterol, such as found in NPCD, SCAP dissociates from SREBP which is therefore broken down by Golgi proteases. A SREBP fragment then migrates to the nucleus (Fig. 1, ❸) Brown and Goldstein 1998) where it acts as a transcription factor and up-regulates the enzymes involved in cholesterol synthesis and uptake (Brown and Goldstein 1998) (Fig. 1, ❹). Thus, NPCD produces a cellular excess of cholesterol but the defect in its lysosomal export fools the cell into believing that it is actually experiencing a cholesterol deficit. The cell responds accordingly by making more cholesterol and attempting to gather more from the extracellular medium (Liscum and Faust 1989; Reddy *et al.* 2006).

For reasons that have yet to be fully explained (but were alluded to briefly above Rocha *et al.* 2009; Garrity *et al.* 2016)) NPCD lysosomes have lower $Ca^{2+}$ content than normal (Lloyd-Evans *et al.* 2008; Xu *et al.* 2012; Visentin *et al.* 2013) and thus calcium efflux is impaired (Shen *et al.* 2012; Höglinger *et al.* 2015) (Fig. 1, ❺). This in turn leads to a reduced activity of the cytosolic phosphatase calcineurin (Medina *et al.* 2015) (Fig. 1, ❻) which hydrolyses transcription factor EB (TFEB) (Napolitano and Ballabio 2016) rendering it active (Fig. 1, ❼). Thus there is less active TFEB to translocate to the nucleus. TFEB up-regulates the CLEAR network (Sardiello *et al.* 2009; Palmieri *et al.* 2011) of genes necessary for lysosomal function and also enhances autophagy (Settembre *et al.* 2011) and lysosomal exocytosis Medina *et al.* 2011) (Fig. 1, ❽) which lead to cellular off-load of toxic substance and their metabolites. Thus, the consequences of reduced lysosomal calcium in NPCD is a reduction in all these processes at the transcriptional level as well as through the vesicle fusion defect discussed above. This is reinforced by the over-activation of MAP kinase in NPCD (Sawamura *et al.* 2001; Sawamura *et al.* 2003) (which occurs for reasons to be discussed later) as one report makes this enzyme responsible for phosphorylating TFEB keeping it inactive (Nada *et al.* 2009) (Fig. 1, ❾). (A variant account (Castellano *et al.* 2017) makes the nutrient sensing complex mTORC1 the kinase for TFEB though the impact of cholesterol storage on mTOR status is still debated (Pacheco *et al.* 2007; Xu *et al.* 2010a; Castellano *et al.* 2017)). MAPK is also known to phosphorylate and thereby activate $PLA_2$ (Fig. 1, ❿) which in turn reduces the action of the transcription factor liver X receptor (LXR, Fig. 1, ⓫) and reduces expression of one of its target genes the cell surface cholesterol exporter ABCA1 (Fig. 1, ⓬) (Choi *et al.* 2003; Wang *et al.* 2007). Hence, cholesterol is retained in the cell and, again, the net result of storage of excess cholesterol in the lysosome is to make the cell behave as though it were suffering a cholesterol deficit.

### From cholesterol storage to cell death

As significant as these processes are, we may question whether the errors are serious enough in NPCD to account for the progressive cell death seen in patient neurones. This can be explained by a complex series of events shown in Fig. 2 (the * suffix represents a protein in its activated state).

Along with cholesterol NPCD endolysosomes accumulate other lipids including sphingomyelin (SM) (Te Vruchte *et al.* 2004; Tharkeshwar *et al.* 2017). These can permeabilize the lysosomal limiting membrane (Fig. 2, ❶); Chung *et al.* 2016; Gabande-Rodriguez *et al.* 2014; Amritraj *et al.* 2013) the glycocalyx which lines the membrane and protects it from the harsh degradative environment of the lysosomal interior is also altered in NPCD (Kosicek *et al.* 2018). Such perforations allow molecules usually localized to the lysosomal interior to escape into the cytosol; among these are the cathepsin proteases (Fig. 2, ❷) which have been shown to damage mitochondria (Fig. 2, ❸) (Cirman *et al.* 2004; Amritraj *et al.* 2013). Mitochondrial damage can also result indirectly from a cellular state known as oxidative stress. Oxidative stress is a harmful increase in reactive oxygen species (ROS, hydrogen peroxide, and the superoxide and hydroxide radicals) caused in turn by their increased generation (often resulting from mitochondrial leak, Fig. 2, ❹) or decreased detoxification pathways (such as catalase in peroxisomes). Increased ROS have been observed in NPCD cells (Koh *et al.* 2006; Klein *et al.* 2011; Kennedy *et al.* 2014) (with patient biochemistry also being affected Fu *et al.* 2010; Ribas *et al.* 2012)) which leads by an unknown

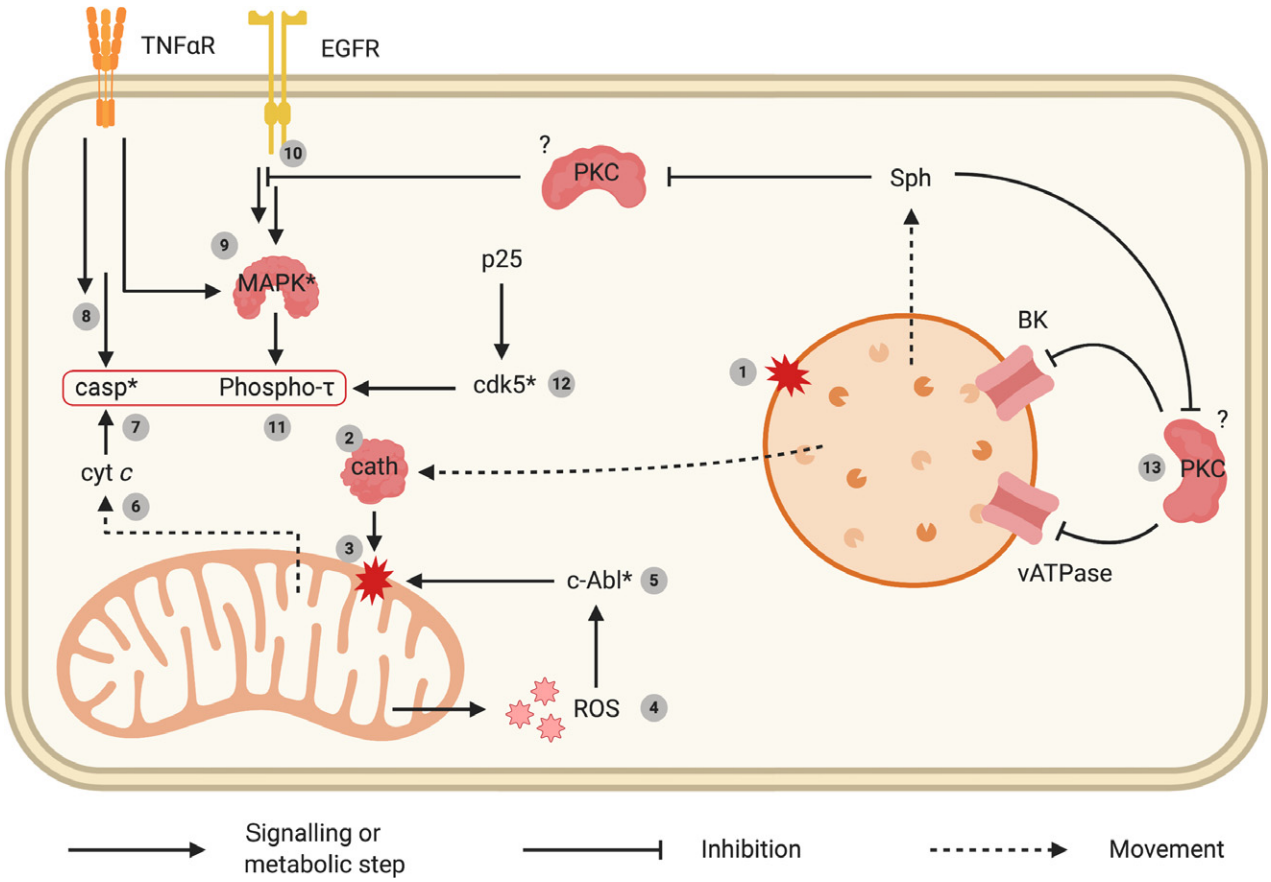


**Fig. 2** From cholesterol storage to cell death Dysfunction in NPCD cells activates events that ultimately result in cell death. For details see text.

mechanism to the activation of the kinase c-Abl (Fig. 2, ❺) (Sun *et al.* 2000; Alvarez *et al.* 2004; Klein *et al.* 2011) and thereby to mitochondrial damage (Ito *et al.* 2001). Whether initiated either by cytosolic cathepsins or by activated c-Abl, mitochondrial damage leads to release of respiratory chain protein cytochrome c (cyt *c*) from mitochondria (Fig. 2, ❻) (Ito *et al.* 2001; Cirman *et al.* 2004; Amritraj *et al.* 2013). This triggers the intrinsic apoptotic pathway (Huang *et al.* 2006) resulting in the activation of the caspase proteolytic cascade (Fig. 2, ❼) (Cai *et al.* 1998) which ultimately dismantles key cellular components including the nuclear envelope and leads to cell death. The extrinsic apoptotic pathway, triggered by binding of TNFα at its receptor (Fig. 2, ❽), is also up-regulated in NPCD (Wu *et al.* 2005). Accordingly, both a c-Abl inhibitor Alvarez *et al.* 2008) and an anti-TNFα antibody (Vincent *et al.* 2010) have demonstrated symptomatic improvement in NPCD mice.

A second pathway leads to cell death: in NPCD mitogen activated protein kinase (MAPK) is found activated (Sawamura *et al.* 2001; Sawamura *et al.* 2003) (Fig. 2, ❾). The reasons for this are unclear, though MAPK can be activated by binding of TNFα to its receptor Sabio and Davis 2014) and this pathway is up-regulated in NPCD (Wu *et al.* 2005) MAPK activation by this means has not yet been explicitly demonstrated for this disease. More commonly MAPK is regarded as being on the EGFR pathway where protein kinase C (PKC) normally serves as a brake (Hunter *et al.* 1984; Livneh *et al.* 2015) (Fig. 2, ❿). Sphingosine (Sph), another lipid that accumulates in NPCD, (Lloyd-Evans *et al.* 2008) may (Hannun and Bell 1987; Rodriguez-Lafrasse *et al.* 1997) or may not (Bazzi and Nelsestuen 1987; Edsall *et al.* 1998) inhibit PKC ($IC_{50}$ at least 1 μM) so if enough Sph escapes the lysosome to effect inhibition, itself an open question, then the brake on the EGFR pathway may be removed leading to activated MAPK. Alternatively, localization may play a role. In rat brain synapse PKC has been found associated with cholesterol and AnxA6 (Orito *et al.* 2001). If the AnxA6 is diverted to lysosomes, as it is in NPCD, (Te Vruchte *et al.* 2004) then the PKC may also be removed from proximity to the cell membrane and so to EGFR. One of the targets of aberrantly activated MAPK is the cytoskeletal protein tau (τ) and so in NPCD this activation leads to hyperphosphorylated tau (Fig. 2, ⓫) (Sawamura *et al.* 2001; Sawamura *et al.* 2003) possibly consistent with the finding of increased tau in the CSF of NPCD patients (Mattsson *et al.* 2011a). Fibrillary tangles

similar to those found in Alzheimer's disease therefore form in NPCD cells; (Auer *et al.* 1995) neuronal cell death results. Consistently, in NPCD cells hyperphosphorylation was reduced *in vitro* on treatment with a MAPK inhibitor (Sawamura *et al.* 2003) An alternative pathway to hyperphosphorylated tau starts with the observation that the protein p25, a proteolytic fragment of p35, is increased in NPCD. p25 serves as an activator of cyclin dependent kinase 5 (cdk5, Fig. 2, ⓬) which then hyperphosphorylates tau; (Bu *et al.* 2002) cdk5 inhibition led to reduced phosphorylation and symptomatic improvements in a mouse model of NPCD (Zhang *et al.* 2004).

None of the preceding paragraph can be regarded as uncontroversial. When MAPK inhibition was translated from *in vitro* to a mouse model enzyme activity was indeed reduced, but tau phosphorylation was increased and no clinical improvement could be observed (Zhang *et al.* 2008). However, selectivity in kinase inhibition is notoriously hard to achieve and the MAPK inhibitor used in both studies (Sawamura *et al.* 2003; Zhang *et al.* 2008) hits a range of enzymes; (Davies *et al.* 2000b) it is therefore difficult to be clear on the origins of the observed effects. Similarly neither of the cdk inhibitors used (Zhang *et al.* 2004) is selective for cdk5 (data from the ChEMBl database (Gaulton *et al.* 2017)). The genetic knockout of p35, the cdk5-activating protein, would be expected to give more targeted effects but did not result in the expected clinical improvements in NPC mice (Hallows et al. 2006).

Nonetheless, PKC activation has been reported by two groups to result in improvements in NPCD cell culture models (Tamari *et al.* 2013; Peter *et al.* 2017) which was attributed to a pathway involving rab9 and the cytoskeletal protein vimentin (see also Walter *et al.* 2003; Walter *et al.* 2009)); the possible involvement of tau was not examined. None of the studies cited in this section investigates the idea that PKC has a potential role in the regulation of vATPase (Nanda *et al.* 1992) and the lysosomal potassium channel BK (Fig. 2, ⓭) (Zhou *et al.* 2010).

### Mitochondria

Mitochondrial damage (Fig. 2, ❸) raises the expectation of metabolic dysfunction in NPCD and indeed reduced mitochondrial potential, (Yu *et al.* 2005; Visentin *et al.* 2013) reduced oxygen consumption, (Kennedy *et al.* 2014; Torres *et al.* 2017) reduced ATP production (Yu *et al.* 2005) and increased levels of lactate (Kennedy *et al.* 2014) have all been reported alongside increased ROS. (Koh *et al.* 2006; Klein *et al.* 2011; Kennedy *et al.* 2014) Most workers attribute this respiratory impairment not to loss of cyt *c*, or structurally aberrant mitochondria more generally, but to excess mitochondrial cholesterol poisoning the organelle. This hypothesis is superficially appealing and also consistent with mitochondrial dysfunction induced by cholesterol overfeeding in hepatic (Domínguez-Pérez *et al.* 2019) and pancreatic (Asalla *et al.* 2016) cells. Perhaps significantly, increased contacts between mitochondria and endosomes, and correspondingly decreased contacts between ER and endosomes, have recently been reported in NPC1-deficient cells (Höglinger *et al.* 2019). However, this idea is also problematic as mechanisms for cholesterol-induced mitochondrial toxicity have been postulated (Torres *et al.* 2017; Solsona-Vilarrasa *et al.* 2019) but not yet proved and an alternate explanation has recently been offered (Yambire *et al.* 2019). Additionally mitochondria play a role in cellular cholesterol homeostasis for they house Cyp27A1 an enzyme responsible for converting cholesterol to 27-hydroxycholesterol, a ligand of the liver X receptor (LXR). (Allen *et al.* 2013) Agonism of this receptor in turn signals increased expression of the cell surface cholesterol exporter ABCA1. Consequently, high levels of cholesterol at the mitochondrion, postulated to account of respiratory dysfunction in NPCD, are a signal that the cell has excess cholesterol and will lead to export of this lipid via increased expression of ABCA1. In fact ABCA1 expression is reduced in NPCD (Choi *et al.* 2003; Wang *et al.* 2007) (although other studies disagree Reddy *et al.* 2006; Vivas *et al.* 2019) and, as we shall see later, LXR agonists have enjoyed some success as therapies. (The role, if any, of SREBP in regulating ABCA1 is unclear with contradictory results having been reported Zeng *et al.* 2004; Wong *et al.* 2006). The issue could perhaps be settled if cholesterol in control and disease mitochondria could be quantitated, but this requires isolation of clean and complete mitochondrial samples. This problem is noted for its difficulty (Kappler *et al.* 2016) and not all NPCD studies that work with isolated mitochondria assess the purity of this cellular fraction. Those that do report increased mitochondrial cholesterol (Yu *et al.* 2005; Charman *et al.* 2010; Kennedy *et al.* 2014; Balboa *et al.* 2017a). Intriguingly, work with isolated mitochondria from healthy cells (Yu *et al.* 2005; Ziolkowski *et al.* 2010) has reported a decrease in respiratory function on cholesterol lowering which, when taken together with the Niemann-Pick work, may suggest that mitochondria have only a narrow range of tolerable cholesterol levels.

If excess cholesterol poisons mitochondria in NPCD how does excess endolysosomal cholesterol find its way to mitochondria instead of the ER or Golgi (Garver *et al.* 2002a; Frolov *et al.* 2003). It has been hypothesized that lysosomal luminal protein NPC2 transports cholesterol to the LEL outer membrane in a manner independent of NPC1 (Kennedy *et al.* 2012) and from there it is collected by steroidogenic acute regulatory protein D3 (StARD3, also known as MLN64) (Charman *et al.* 2010; Balboa *et al.* 2017a) and inserted into the mitochondrial membrane. This idea is seemingly inconsistent with the widely held model of cholesterol export which sees cholesterol transferred from NPC2 to NPC1 (Subramanian and Balch 2008; Infante *et al.* 2008b) before export and would lead to the expectation that

NPC2-deficient cells would have normal mitochondria. Whilst this issue has not yet been studied in the context of NPC disease, knockdown of NPC2 in liver cells reduced mitochondrial function (Wang *et al.* 2018) suggesting that if cholesterol is the offending lipid in Niemann–Pick then its path from lysosome to mitochondrion remains to be traced fully. We will return to this issue briefly when we discuss secondary lipid manipulation as treatment.

So, starting from a simple failure of metabolite export, NPCD progresses via multiple pathways to affect numerous cellular functions and results ultimately in cell death. This multi-faceted pathology poses an obvious question to those who would treat the disease: which aspect do you target? Perhaps surprisingly, many of the treatment options that have been explored depend upon the sphingolipids which co-accumulate with cholesterol in the NPCD lysosome. Thus it is important to understand their metabolism – for a recent review see (Sandhoff and Sandhoff 2018).

### Sphingolipid metabolism

Sphingolipid synthesis *in vivo* begins with the condensation of serine with a long-chain acyl-CoA, usually palmitoyl, mediated by serine palmitoyl transferase. The reduction in the ketone group thereby generated precedes acylation of the nitrogen with a second long-chain acyl-CoA mediated by ceramide synthase before oxidation to give a carbon-carbon double bond yields ceramide. These steps happen in the ER (Tidhar and Futerman 2013). Ceramide is then transported to the Golgi in a vesicular manner and also by ceramide transfer protein (CERT, also known as StARD11) (Giussani *et al.* 2008). Translocation to the luminal face of the Golgi membrane allows addition of a zwitterionic head group to give sphingomyelin (SM). Ceramide remaining on the cytoplasmic face can be glycosylated to give glucosylceramide (GlcCer) by the requisite synthase GCS (Futerman and Pagano 1991; Jeckel *et al.* 1992). GlcCer is thus the simplest glycosphingolipid (GSL). GlcCer can then be transferred to the opposite face of the Golgi membrane, likely by an indirect mechanism involving the ER, (D'Angelo *et al.* 2013) where elaboration to higher GSLs such as gangliosides occurs. GlcCer is also transported to the cell membrane by a non-vesicular route (Halter *et al.* 2007) mediated by glycolipid transfer protein (GLTP) and phosphatidylinositol-four-phosphate adapter protein 2 (FAPP2, also known as PLEKHA8) where it eventually emerges on the cell surface (Halter *et al.* 2007). This renders it susceptible to endocytosis and therefore metabolism. Thus on an internal lysosomal membrane GlcCer is hydrolysed to ceramide by glucocerbrosidase (GBA1) (Gillard *et al.* 1998). Ceramide is further catabolized by acid ceramidase to sphingosine (Sph) which is returned to the ER by an unknown mechanism and processed to regenerate ceramide. A minor sub-population of GlcCer on the cytosolic face of membranes can be hydrolysed by specific enzyme GBA2.

Most obviously the localization of sphingolipid metabolic enzymes means the two sides of the lipid bilayers which form cell and organelle membranes are inequivalent (van Meer *et al.* 2008). Ceramide is glycosylated only on the cytosolic face of the Golgi membrane, and subsequent processes preserve the asymmetry this induces. For example GLTP and FAPP2 transport GlcCer to the cell membrane (Halter *et al.* 2007) where they are believed to interact with existing lipids (Mattjus 2009) to insert GlcCer. This insertion necessarily occurs only on the cytosolic (inner) leaflet. Although other sphingolipids may also exist on both sides of the membrane (e.g., SM), GlcCer has been shown to be rapidly metabolized and transported via a non-vesicular pathway on the cytosolic side (Warnock *et al.* 1994).

## Treatment

### Expression

Histone deacetylases (HDACs) are a family of enzymes involved in regulating gene transcription and are over-expressed in NPCD (Munkacsi *et al.* 2011) possibly as a result of c-Abl activation (Fig. 2, ❺) (Contreras *et al.* 2016). Consequently inhibition of HDACs leads to increased expression of NPC1, (Pipalia *et al.* 2011) enough of which can then be transported to the lysosome where sufficient function is preserved to export stored cholesterol and normalize levels of this lipid (Munkacsi *et al.* 2011; Pugach *et al.* 2018). From testing various small molecule inhibitors with different selectivity profiles HDAC1 and −2 were tentatively identified as the key enzymes to target (Pipalia *et al.* 2011). Experiments in mice have thus far failed to confirm the *in vitro* promise of such compounds (Alam *et al.* 2018).

After translation to protein newly synthesized NPC1 is transported from the ER to the lysosome chaperoned by Hsp70; (Nakasone *et al.* 2014) mutant protein misfolds and is targeted for degradation (Gelsthorpe *et al.* 2008). Supplying a small molecule scaffold of the correct shape can cause mutant protein to fold correctly and thereby escape proteolysis. While the structural requirements of such an agent have been defined, (Ohgane *et al.* 2014) and correct protein localization demonstrated, (Ohgane *et al.* 2013) studies have not progressed to disease modification.

A recent approach seeks to correct protein misfolding by manipulating ER calcium (Yu *et al.* 2012). While this has been successful in a mouse model of another LSD (Liou *et al.* 2016) its potential in NPCD is currently unclear given that pathology may depend on calcium in multiple ways.

### Cholesterol

Trapping excess cholesterol in the lysosome fools the NPCD cell that it is experiencing a cholesterol deficit. It is therefore possible that therapies aimed at cholesterol reduction will be ineffective. Indeed when lipid-lowering

agents were tried in patients, levels of plasma and hepatic cholesterol fell, but no impact on disease was reported (Patterson *et al.* 1993). In NPCD mice nifedipine (a $Ca^{2+}$-channel blocker, postulated also to induce cholesterol efflux) and probucol (an inhibitor of cell surface cholesterol exporter ABCA1) led to reductions in hepatic cholesterol but no effect on disabling neurological symptoms (Erickson *et al.* 2000). (Given the later finding that ABCA1 is down-regulated in NPCD Choi *et al.* 2003; Wang *et al.* 2007) this is not surprising.) Lipid lowering agent clofibrate failed to reduce cholesterol levels in disease fibroblasts, (Beheregaray *et al.* 2003) though given the pleiotropic effects of this class of drugs this finding might have been expected. Statins have recently been revisited and found ineffective in iPSC-derived neurones, (Yu *et al.* 2014) although showing some promise in oligodendrocyte culture (Yang *et al.* 2018).

A subtler approach to cholesterol reduction is an agonist of the liver X receptor (LXR) which leads to an increase in ABCA1 levels in turn causing cholesterol offload in NPC1-deficient cells (Boadu *et al.* 2006; Boadu *et al.* 2012). This increased the lifespan of NPCD mice though offered only a very slight improvement in neurological symptoms (Repa *et al.* 2007).

Cyclodextrins (CDs) are large hydrophilic molecules containing a hydrophobic cleft and may solubilize lipophilic molecules including cholesterol. A range of studies has demonstrated the effectiveness of CDs in treating NPCD, although not, as might first be expected, by extracting cholesterol from membranes (Chen *et al.* 2010). Rather CDs are endocytosed (Chen *et al.* 2010; Rosenbaum *et al.* 2010) where they sequester excess cholesterol in the LEL and return it to circulation – cholesterol is thus exocytosed, (Chen *et al.* 2010) reduced at the lysosome (Rosenbaum *et al.* 2010) and increased at the ER; (Abi-Mosleh *et al.* 2009) cholesterol synthesis is reduced (Liu *et al.* 2009) (An alternative narrative makes cholesterol normalization dependent on corrected autophagy (Dai *et al.* 2017). While an explanation of the reasons behind this is not offered it is noteworthy that the sphingolipid field has produced similar findings on the interdependence of the two processes (Young *et al.* 2016; Lima *et al.* 2017)). Mitochondrial dysfunction is also normalized by cyclodextrin treatment, (Yu *et al.* 2005) though these experiments were conducted on isolated mitochondria which questions whether this approach would be successful in whole cells. CDs have been successfully used in mouse models of NPCD (Davidson *et al.* 2009; Liu *et al.* 2009; Ramirez *et al.* 2010) although a major drawback is their inability to cross the blood–brain barrier (Calias 2017). Use in patients thus requires intrathecal administration; this technique underpins the initial success (Ory *et al.* 2017; Berry-Kravis *et al.* 2018) of a small clinical trial (NCT01747135) Other studies (e.g., NCT03471143) are planned.

### LEL calcium

As we have already seen NPCD cells have a deficit in endolysosomal $Ca^{2+}$, as demonstrated by multiple studies (Lloyd-Evans *et al.* 2008; Xu *et al.* 2012; Visentin *et al.* 2013) (although there has been some disagreement (Shen *et al.* 2012)). This might explain the defects in endocytosis and autophagy, both of which depend on $Ca^{2+}$-efflux from the lysosome. Thus inhibiting $Ca^{2+}$-uptake by the ER might increase cytosolic $Ca^{2+}$, allow lysosomal stores to refill and so restore LEL function. Accordingly curcumin, an inhibitor of the SERCA calcium pump, restored aberrant endocytosis *in vitro* and improved lifespan in NPCD mice (Lloyd-Evans *et al.* 2008). While neurological defects in the murine model were unaffected by curcumin monotherapy, (Borbon *et al.* 2012; Williams *et al.* 2014) the benefits of other treatments were amplified (Williams *et al.* 2014).

Calcium is also a key intermediary in the pathway of adenosine$_{2A}$ receptor ($A_{2A}R$) agonists the only extra-cellular target successfully investigated to date. The success of this approach in correcting both lysosomal and mitochondrial defects in a whole cell model (Visentin *et al.* 2013; Ferrante *et al.* 2016) marks it as unique. Pharmacological experiments suggest a downstream effect of $A_{2A}R$ agonism is PKA activation (Ferrante *et al.* 2016) consistent with the classical pathway of this GPCR (though earlier work in fibroblasts invoked the ERK class of MAP kinases instead (Visentin *et al.* 2013)). The pathway between PKA activation and $Ca^{2+}$-mobilization is not explored, nor is the idea PKA can activate CFTR and induce lysosomal re-acidification (Chang *et al.* 1993; Folts *et al.* 2016). The finding that $A_{2A}R$ agonism can lead to cholesterol efflux via increased ABCA1 expression (Bingham *et al.* 2010) is likewise neglected. $A_{2A}R$ agonists in NPCD mice give small improvements in neurological symptoms and lifespan (Ferrante *et al.* 2018).

### Sphingolipids

Some lysosomal storage disorders result from an inability to metabolize gangliosides correctly; these lipids thus accumulate. The observations that NPCD patients have a secondary accumulation of gangliosides and present clinically similar symptoms to patients with a deficiency in ganglioside metabolism, prompted the notion that gangliosides could be pathological in NPCD (Zervas *et al.* 2001). Given the absence of small molecule inhibitors of ganglioside synthases a logical treatment choice would therefore be to inhibit synthesis of their simpler glycolipid precursor GlcCer; indeed GCS inhibitor N-butyldeoxynojirimycin (NB-DNJ) proved successful at alleviating symptoms in both cat and mouse models of the disease (Zervas *et al.* 2001). The first report of the effective treatment of a human patient emerged in 2004 (Lachmann *et al.* 2004) and was followed by clinical trials which demonstrated stabilization of disease progression and even some improvements (Patterson *et al.* 2007; Pineda *et al.* 2009). These developments led to the approval of NB-DNJ (miglustat,

Zavesca®, Actelion Pharmaceuticals Ltd, Switzerland reviewed (Pineda *et al.* 2019)) in Europe in 2009. Subsequent studies have questioned miglustat's mode of action. While the improvement in a murine model was confirmed, brain lipid analysis showed an increase in GlcCer (and no effects on gangliosides GM2 or GM3) – inconsistent with its postulated action as a GCS inhibitor (Nietupski *et al.* 2012). Off-target effects at GlcCer cytoplasmic hydrolase GBA2 were suggested and *in vitro* work later confirmed that miglustat was in fact a more potent inhibitor of this enzyme than of the synthase (Ridley *et al.* 2013). Use of a rationally designed GBA2 inhibitor (Overkleeft *et al.* 1998) has recently been effective in treating NPCD mice (Marques *et al.* 2015) while genetic deletion of GM2 or −3 synthases in murine models failed to yield improvements (Liu *et al.* 2000; Lee *et al.* 2014). This is consistent with miglustat being clinically effective but not altering GM2 or −3 levels and suggests that these gangliosides are not the toxic lipids in NPCD. Accordingly, the sub-population of GlcCer on the cytosolic face of membranes ('cytosolic GlcCer') emerges as an important factor in NPC disease. We have recently demonstrated that cytosolic GlcCer may be responsible for regulating vATPase and hence that GBA2 inhibition results in correction of defects in both lysosomal acidification and endocytic trafficking in NPCD (Wheeler *et al.* 2019a). Potentially this ties in well with the ideas that NPC1 is a sphingosine exporter (Lloyd-Evans *et al.* 2008; Höglinger *et al.* 2015; Höglinger *et al.* 2017; Wheeler *et al.* 2019b) and that Sph inhibits GBA2 (Schonauer *et al.* 2017). Thus reduced Sph export removes an endogenous negative regulator of GBA2 leading to a reduction in cytosolic GlcCer and consequently reduced vATPase activation and increased LEL pH (Chakraborty *et al.* 2017; Tharkeshwar *et al.* 2017; Wheeler *et al.* 2019a).

There are some possible commonalities here with Gaucher disease which results from loss of function mutations of the lysosomal GlcCer hydrolase GBA1. Both Gaucher and NPCD feature lysosomal storage of GlcCer, both have up-regulated GBA2 (Burke *et al.* 2013; Marques *et al.* 2015) and both are clinically treated with GBA2 inhibitor miglustat. Thus deficient levels of cytosolic-facing GlcCer may be toxic (rather than excessive levels of lipids in the lysosome) and these can be corrected through inhibition of GBA2 – in NPCD fibroblasts nanomolar concentrations of GBA2 inhibitor AMP-DNJ led to large increases in total GlcCer (Wheeler *et al.* 2019a). Whether this is genuinely the pathway, and if so whether it can be generalized, remains to be seen. The possibility that miglustat treatment may reduce both tau and amyloid accumulation in NPCD patients (Mattsson *et al.* 2011a; Mattsson *et al.* 2011b) suggests that miglustat repurposing and clarification of miglustat targets (GBA2 and GCS) might be fruitful future avenues (Wheeler *et al.* 2019a).

Another important sphingolipid pool is that of sphingomyelin (SM) in the intra-lysosomal membranes mentioned above. These membranes are rich in specialized lipid bis(monoacylglycero)phosphate (BMP) also known as lysobisphosphatidic acid (LBPA), (Kobayashi *et al.* 1999; Chevallier *et al.* 2008). SM is degraded at these membranes by acid sphingomyelinase (aSMase), a process which depends on the interaction of the enzyme with BMP (Reagan *et al.* 2000; Tamura *et al.* 2006). The activity of aSMase is reduced in NPCD (Reagan *et al.* 2000; Tamura *et al.* 2006) despite normal expression (Reagan *et al.* 2000) and localization (Tamura *et al.* 2006). This contributes to high levels of SM in NPCD endolysosomes which, as noted above, can permeabilize the limiting membrane (Amritraj *et al.* 2013; Gabande-Rodriguez *et al.* 2014; Chung *et al.* 2016) leading ultimately to apoptosis. Hsp70 stabilizes the aSMase-BMP interaction (Kirkegaard *et al.* 2010) and thus dosing recombinant Hsp70 corrects errors in a murine model of NPCD (Kirkegaard *et al.* 2016) (These mice entirely lack NPC1 so it was not possible to assess whether Hsp70 also chaperoned transport of mutant NPC1 to endolysosomes (Nakasone *et al.* 2014) thus giving sufficient NPC1 activity). The effect could be recapitulated by synthetic Hsp70 inducer arimoclomol (Kirkegaard *et al.* 2016). A human clinical trial (NCT02612129) with this agent has recently concluded with positive results. This research raises other questions. Both SM (Puri *et al.* 2003) and BMP (Kobayashi *et al.* 1999; Chevallier *et al.* 2008) are reported to associate strongly with cholesterol at the intra-luminal membranes; cell, model membrane and computational studies all suggest that SM has an inhibitory effect on cholesterol transfer (Abdul-Hammed *et al.* 2010; Oninla *et al.* 2014; Enkavi *et al.* 2017) and increasing aSMase activity results in increased cholesterol export in normal cells (Gallala *et al.* 2011). More surprisingly, but consistently, increasing aSMase activity decreased lysosomal cholesterol in NPCD cells (Devlin *et al.* 2010). Comparable findings have recently been reported with BMP (Moreau *et al.* 2019).

## Concluding remarks

The survey of treatments arrived at the point where it appears to be possible to reduce lysosomal cholesterol in NPC1 deficient cells by doing nothing more than altering the population of another lipid inside the lysosome (Devlin *et al.* 2010; Kirkegaard *et al.* 2010; Gallala *et al.* 2011). Other work finds that the same result can be achieved by over-expression of rab7 (Choudhury *et al.* 2002), use of a viral rab7 equivalent (Cianciola and Carlin 2009; Cianciola *et al.* 2013), by increasing expression of ABCA1 (Boadu *et al.* 2006; Boadu *et al.* 2012), by inhibiting O-glycosylation of lysosomal membrane proteins (Li *et al.* 2015) or forcing ER-endosome contact sites (Höglinger *et al.* 2019). At the very least this suggests that NPC1 is not required for endolysosomal cholesterol export (though NPC2 may be Boadu *et al.* 2012; Kennedy *et al.* 2012; Cianciola *et al.* 2013)). A more radical version of this hypothesis argues on kinetic grounds (Lloyd-Evans *et al.* 2008) that cholesterol storage in NPCD

is not primary but secondary and therefore the main or only function of NPC1 is not cholesterol export but some other form of endolysosomal regulation. The pathway postulated above linking NPC1, Sph, cytosolic GlcCer, and LEL pH may stimulate productive studies along these lines, as may the finding that NPC1 regulates other lysosomal proteins including cathD (Macías-Vidal *et al.* 2016).

## Acknowledgements

The authors are grateful to Niemann-Pick Research Foundation for funding. Figures were re-drawn by Marco Bazelmans in BioRender (https://biorender.com/) on the basis of a draft provided by the authors.

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