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Acidic Ca²⁺ stores in neurodegeneration

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Abstract

Lysosomes have emerged in the last decade as an immensely important intracellular site of Ca²⁺ storage and signalling. More recently there has been an increase in the number of new ion channels found to be functional on lysosomes and the potential roles that these signalling pathways might play in fundamental cellular processes are being uncovered. Defects in lysosomal function have been shown to result in changes in lysosomal Ca²⁺ homeostasis and ultimately can result in cell death. Several neurodegenerative diseases, from rare lysosomal storage diseases through to more common diseases of ageing, have recently been identified as having alterations in lysosomal Ca²⁺ homeostasis that may play an important role in neuronal excitotoxicity and ultimately cell death. This review will critically summarise these recent findings.

Introduction

In recent years, acidic endo-lysosomal Ca²⁺ stores have emerged as a major component of intracellular Ca²⁺ signalling in mammalian cells (Patel & Cai, 2015). Ca²⁺ is one of the most important signalling molecules inside and outside of cells and is an important component of the triggering of fertilization, cell growth and motility and ultimately cell death (Berridge, Lipp et al., 2000). Within cells Ca²⁺ levels are tightly regulated with nM to low μ M levels in the cytosol and high μ M to low mM levels within some organelles such as the endoplasmic reticulum and acidic stores such as late endosomes and lysosomes (Lloyd-Evans & Platt, 2011). Changes in cytosolic Ca²⁺ levels, via Ca²⁺ release from intracellular organelles or Ca²⁺ entry from the extracellular fluid, triggers cellular changes (Berridge et al., 2000). Ca²⁺ channels present on the plasma membranes or intracellular organelles act in response to extracellular stimuli (primary messengers) or intracellular second messengers such as nicotinic acid adenine dinucleotide phosphate (NAADP) to either trigger Ca²⁺ entry into the cell or Ca²⁺ exit from organelles (Galione & Churchill, 2002). In order to restore cytosolic Ca²⁺ levels back to their normally low levels a variety of pumps and transporters exist that transport Ca²⁺ out of the cell and into intracellular organelles (Brini & Carafoli, 2011, Mekahli, Bultynck et al., 2011). Failure to maintain cytosolic Ca²⁺ levels can lead to continuously elevated Ca²⁺ that can trigger excitotoxicity and cell death (Berridge et al., 2000, Mekahli et al., 2011). One intracellular organelle that appears to regulate global changes in Ca²⁺ signalling and which may act to modulate excitotoxic changes in cytosolic Ca²⁺ is the lysosome (Lloyd-Evans & Platt, 2011, Lloyd-Evans, Waller-Evans et al., 2010).

This review aims to highlight the role of the lysosome as a Ca^{2+} store that is involved in the pathogenesis of multiple neurodegenerative diseases.

The need for Ca^{2+} in the endo-lysosomal system

The endo-lysosomal system is an essential series of distinct compartments found in all nucleated cells (Luzio, Pryor et al., 2007). Endocytosis is the process by which material from the extracellular fluid enters the cells, either by binding to receptors or by fluid phase endocytosis, via budding from the plasma membrane. From here, vesicles fuse with the early endosomes and deposited cargo is transported from early endosomes to the late endosome whereas most receptors are recycled back out of the cell via the endocytic recycling compartment. Cargo in the late endosome is usually delivered to lysosomes, the terminal point of the endocytic system, for degradation (Luzio, Parkinson et al., 2009). Lysosomes have an acidic lumen, ranging from pH 4-5 dependent on cell type (Lloyd-Evans, Morgan et al., 2008), and are comprising numerous highly glycosylated transmembrane proteins that form a protective luminal glycocalyx to prevent self digestion by the lysosomes own high content of acidic hydrolases (Schulze, Kolter et al., 2009). Lysosomes are the main recycling centre of the cell with the products of this degradation of macromolecules being redistributed to endoplasmic reticulum (ER) or Golgi by vesicular transport or pumped into the cytosol via the action of lysosomal membrane transporters (Saftig & Klumperman, 2009). Lysosomes are also essential in the degradation of defective organelles, particularly mitochondria, and mis-folded proteins, all of which enter the lysosomal system through either autophagic vacuole fusion with late endosomes or chaperone mediated autophagy (Platt, Boland et al., 2012).

All of these processes of transport along the endocytic system, membrane recycling and autophagic vacuole fusion are Ca^{2+} dependent processes (Lloyd-Evans et al., 2010, Piper & Luzio, 2004). An early breakthrough in our understanding of how Ca^{2+} regulates endocytic processes was the finding that localised Ca^{2+} release is required for the fusion of lysosomes with late endosomes. Using a fast Ca^{2+} chelator, BAPTA, versus the slower acting EGTA, it was found that fusion between purified late endosomes and lysosomes could be inhibited. Furthermore, the amount of Ca^{2+} required to induce fusion between these organelles was in the range of 0.1-10 μM . Taken together, this would suggest a rapid localised Ca^{2+} release most likely from a substantial intraorganellar Ca^{2+} store (Pryor, Mullock et al., 2000). Further understanding of the role of Ca^{2+} in regulating endocytosis came from the discovery that the Ca^{2+} dependent phospholipid binding protein Annexin A2, which mediates cargo delivery between membranes, is required for normal transport of vesicular cargo between early endosomes and late endosomes (Lloyd-Evans et al., 2008, Mayran, Parton et al., 2003). Disruption in endosomal Ca^{2+} handling, the mechanisms of which are discussed in more detail below, can lead to severe disruption in endocytic and cellular function (Lloyd-Evans & Platt, 2011, Lloyd-Evans et al., 2010). Enhanced Ca^{2+} release from lysosomes in response to the second messenger NAADP has been shown to lead to defective endocytosis and recycling of lipids out of late endosomes and lysosomes resulting in their accumulation within enlarged lysosomes (Ruas, Rietdorf et al., 2010). Disruption of Ca^{2+} signalling is also known to lead to defects in autophagic vacuole-lysosome fusion (Gordon, Holen et al., 1993, La Rovere, Roest et al., 2016), another endocytic process which requires localised Ca^{2+}

release from lysosomes (Medina, Di Paola et al., 2015), defects in this signalling result in accumulation of autophagosomes and defective mitochondria within cells (Medina & Ballabio, 2015). Furthermore, defects in endo-lysosomal Ca^{2+} release are coupled to changes in pigmentation (Lin-Moshier, Keebler et al., 2014), potentially as a result of altered lysosomal exocytosis. All of these disruptions in the endocytic system are known to occur in neurodegenerative lysosomal storage diseases and more common diseases of ageing (Lloyd-Evans & Haslett, 2016b), and are discussed in more detail below.

Endo-lysosomes are a major intracellular Ca^{2+} store

Although the largest Ca^{2+} store within the cell is the endoplasmic reticulum (ranging from 0.1-1mM (Bygrave & Benedetti, 1996)), it has become apparent in recent years that the endocytic system, especially the so called acidic stores, namely the late endosomes and lysosomes, contain substantial levels of Ca^{2+} (Christensen, Myers et al., 2002, Lloyd-Evans et al., 2008). It should however be noted that although the intra-organelle Ca^{2+} content within lysosomes is high (see below), volumetrically lysosomes are predicted as being only 2-3% of the total cellular volume (Thoene, 1992). As such their impact on changing whole cell cytosolic Ca^{2+} levels should be minimal in comparison to ER (>10% of the cellular volume (Voeltz, Rolls et al., 2002)) Ca^{2+} release or extracellular Ca^{2+} influx. However, recent studies have indicated that lysosomal Ca^{2+} release can trigger substantial Ca^{2+} release from the ER which in turn has a global effect on elevating cellular Ca^{2+} levels (Kilpatrick, Eden et al., 2013, Morgan, Davis et al., 2013). The observation of high intra-luminal lysosomal Ca^{2+} levels explain the origin of the localised elevations in Ca^{2+} that are required for endo-lysosomal fusion and endocytic vesicle transport discussed above (Pryor et al., 2000). Rather than changes in global Ca^{2+} eliciting endocytic transport and recycling, it would appear that this is regulated instead by Ca^{2+} release from endo-lysosomal stores. This was confirmed by studies using dextran conjugated Ca^{2+} chelators endocytosed into lysosomes that induced lysosomal lipid storage and endocytic trafficking abnormalities, indicating that luminal lysosomal Ca^{2+} is indeed critical for endocytosis (Lloyd-Evans et al., 2008). Whilst extracellular Ca^{2+} levels are high (can range from 1.2-1.8mM in plasma, interstitial fluid and cerebrospinal fluid (Jones & Keep, 1988)), the intra-luminal Ca^{2+} content of early endosomes has been measured as being in the range of 5-30 μM (Gerasimenko, Tepikin et al., 1998). The reason for this discrepancy, which is substantial when one considers the volume of extracellular fluid that continuously enters early endosomes, is the initiation of acidification of the endocytic system at the early endosome level by the action of the H^+ pumping vacuolar ATPase (v-ATPase) (Gerasimenko et al., 1998, Lelouvier & Puertollano, 2011). This process results in loss of Ca^{2+} from the early endosomal lumen to the cytosol, suggested to be mediated by the transient receptor potential mucolipin (TRPML3) ion channel (Lelouvier & Puertollano, 2011). Furthermore, it has been shown that endocytosis contributes very little to the overall Ca^{2+} content of the acidic stores (Christensen et al., 2002). Using dextran conjugated Ca^{2+} probes we and others have shown that it is possible to specifically load the lysosome with these probes through a pulse/chase experiment. By adjusting the K_d of the Ca^{2+} probe for the pH of the lysosome, and ensuring that a low affinity Ca^{2+} probe is used, it is then possible to measure the intra luminal free Ca^{2+} content of this highly acidic organelle. We have found that although there

is some slight differences when comparing cell types, on the whole lysosomal Ca^{2+} appears to be in the range of 500-600 μM (Christensen et al., 2002, Lloyd-Evans et al., 2008), indicating that this is the second largest Ca^{2+} store within the cell. How lysosomal Ca^{2+} levels are maintained is discussed in more detail below, and is summarised in Fig. 1, but we are aware that maintenance of the pH gradient is essential as disruption of the v-ATPases leads to expulsion of Ca^{2+} from lysosomes in a similar manner to that which occurs from the ER following inhibition of the ER Ca^{2+} ATPase (SERCA) with thapsigargin (Christensen et al., 2002, Kilpatrick, Magalhaes et al., 2016a).

Maintenance of endo-lysosomal Ca^{2+} levels; pumps, transporters and channels

Lysosomal Ca^{2+} entry mechanisms

The mechanisms governing Ca^{2+} entry into lysosomes remain largely unknown. Evidence for the presence of an ATPase on lysosomal membranes has emerged from studies into neutrophils and platelets. Platelet dense core granules, organelles very similar to lysosomes, have been shown to contain SERCA3a and its inhibition by TBHQ resulted in a reduction in lysosomal Ca^{2+} content (Lopez, Jardin et al., 2008). Furthermore, studies in neutrophils have shown the presence of a high affinity ATP dependent lysosomal Ca^{2+} uptake pump that was dependent on Mg^{2+} (Klemper, 1985). Studies in purified lysosomes however have shown the presence of ATP independent Ca^{2+} entry that can be inhibited by heavy metals including Zn^{2+} and Mg^{2+} but not by monovalent ions, is also inhibited by L-cystine and is less efficient when the cytosol is acidified (Lemons & Thoene, 1991). This may provide support for the presence of $\text{Ca}^{2+}/\text{H}^{+}$ exchangers on lysosomal membranes similar to the CAX family which have recently been identified on the lysosomes of non-placental mammals (Melchionda, Pittman et al., 2016) or the solute transporter SLC24A5, a K^{+} dependent $\text{Na}^{+}/\text{Ca}^{2+}$ transporter, has been shown to be important in pigmentation in zebrafish and humans and resides on the surface of melanosomes, a lysosome related organelle (Lamason, Mohideen et al., 2005). Ultimately, the identification of pumps and transporters that could fill the lysosome with Ca^{2+} remains a major fundamental area of research for the lysosomal Ca^{2+} field with the majority of evidence pointing towards some form of transport mechanism reliant on pH (Morgan, Platt et al., 2011). However, a recent study has suggested that Ca^{2+} uptake into lysosomes is not dependent on acidification but instead the ER is utilised, via Ca^{2+} release from InsP3 receptors, to fill the lysosome with Ca^{2+} (Garrity, Wang et al., 2016). This study is supported by others indicating that lysosomes selectively sequester Ca^{2+} released from the ER (as opposed to Ca^{2+} entering the cell via store operated pathways) via contact points between these organelles (Lopez Sanjurjo, Tovey et al., 2014, Lopez-Sanjurjo, Tovey et al., 2013). However, these studies also indicate that blocking lysosomal acidification with bafilomycin can perturb the ability of lysosomes to sequester Ca^{2+} released from the ER (Lopez Sanjurjo et al., 2014). All of these studies are in keeping with the findings that alteration in lysosomal pH in familial Alzheimer disease (FAD) cells results in reduced, but not completely diminished, levels of lysosomal Ca^{2+} (Coen, Flannagan et al., 2012, Lee, McBrayer et al., 2015). This is described in more detail below but the defect in lysosomal Ca^{2+} in these cells is believed to be activation of TRPML1, a late endosomal/lysosomal ion channel, triggered by abnormal lysosomal pH

incurring a Ca^{2+} leak via TRPML1 out of lysosomes (Lee et al., 2015). This and other studies would therefore suggest that lysosomal pH can maintain endogenous lysosomal Ca^{2+} levels by controlling lysosomal Ca^{2+} uptake from the ER and also triggering lysosomal Ca^{2+} release (Christensen et al., 2002, Lopez Sanjurjo et al., 2014, Morgan & Galione, 2007). It is also evident that Ca^{2+} release from lysosomes can also induce Ca^{2+} release from the ER (Kilpatrick et al., 2013), this is mediated by defined channels described below.

Lysosomal Ca^{2+} release mechanisms

The same is also true for lysosomal Ca^{2+} release, where a plethora of ion channels are now believed to be involved. The two pore channel 2 (TPC2) and the TRPML1 channel are the two best characterised of these ion channels (reviewed in (Patel & Cai, 2015)) but recent evidence has also emerged suggesting that the purinergic ATP activated cation channel P2X4 and the voltage gated Ca^{2+} channel CACNA1a are also present on lysosomes (Cao, Zhong et al., 2015, Huang, Zou et al., 2014, Tian, Gala et al., 2015). For the purpose of this review we will focus on TPC2 and TRPML1 as these two lysosomal ion channels are the ones most associated with neurodegenerative diseases. TPC2 was originally identified as the channel that transports Ca^{2+} out of lysosomes in response to the most potent intracellular Ca^{2+} releasing second messenger NAADP (Brailoiu, Churamani et al., 2009, Calcraft, Ruas et al., 2009), which had previously been shown to target lysosomes (Churchill, Okada et al., 2002). Based on single channel recordings, TPC2 is known to respond reversibly to NAADP under acidic luminal conditions (pH 4.8) and responds to lower concentrations of NAADP when luminal lysosomal Ca^{2+} concentration is higher, suggesting that luminal Ca^{2+} and pH both regulate TPC activity (Pitt, Funnell et al., 2010). The channel has been shown to be capable of transporting Na^+ (Wang, Zhang et al., 2012) but appears to primarily transport Ca^{2+} (Pitt, Lam et al., 2014) and evidence that TPC2 does not respond to NAADP has been refuted (Ruas, Davis et al., 2015).

In addition to TPC2, the late endosomal/lysosomal system has another well characterised Ca^{2+} permeant channel known as TRPML1. TRPML1 is, at present, the only TRP family member of ion channels exclusively present in the lysosome (TRPM2, a plasma membrane channel, has also been identified on lysosomes (Lange, Yamamoto et al., 2009)) and loss of function is associated with the human lysosomal storage disease mucopolipidosis type IV (MLIV) described in detail below. Initially, TRPML1 was believed to be a candidate NAADP responsive channel (Zhang, Jin et al., 2009), but this was shown not to be the case as overexpression of TRPML1 does not enhance NAADP mediated Ca^{2+} release (whereas TPC2 does (Yamaguchi, Jha et al., 2011)). Subsequently, the endogenous ligand for TRPML1 was identified as being phosphatidylinositol 3,5-bisphosphate ($\text{PI}(3,5)\text{P}_2$) (Dong, Shen et al., 2010), which is also capable of modulating TPC2 activity (Jha, Ahuja et al., 2014, Wang et al., 2012). TRPML1 appears to operate in both late endosomes and lysosomes (Piper & Luzio, 2004, Wong, Li et al., 2012) and is permeant to multiple ions including, Ca^{2+} , Fe^{2+} , Na^+ , K^+ and Zn^{2+} (Cuajungco, Basilio et al., 2014, Dong, Cheng et al., 2008, LaPlante, Falardeau et al., 2002). Functionally, Ca^{2+} signalling mediated by TRPML1 has been linked to the biogenesis of lysosomes and the clearance of autophagic vacuoles via activation of the transcription factor EB (TFEB) (Medina et al., 2015, Medina, Fraldi et al., 2011).

Abnormal lysosomal Ca^{2+} signalling as a contributing factor to neurodegeneration and cellular dysfunction in the lysosomal storage diseases

Defects in lysosomal Ca^{2+} maintenance or signalling have been associated with human diseases, mainly with the lysosomal storage disorders (Lloyd-Evans et al., 2010), a group of predominantly childhood neurodegenerative diseases (Cox & Cachon-Gonzalez, 2012). Lysosomal storage diseases (LSDs), whilst being individually rare, are collectively the most common form of childhood neurodegenerative disease occurring at a combined frequency of 1:5,000 live births and comprise a family of >60 diseases (Cox & Cachon-Gonzalez, 2012). The majority are caused by defects in lysosomal enzymes, but an increasing cohort of ~20 are caused by genetic mutations in genes encoding for lysosomal transmembrane proteins (Lloyd-Evans & Platt, 2010). For almost all of these diseases the exact mechanisms that lead to neuronal cell death remain largely unknown and treatment options for most of the diseases are limited to palliative care (Platt & Jeyakumar, 2008). Work by us and others has led to the emergence of evidence connecting several of these disorders with defects in intracellular Ca^{2+} handling, that in some cases results in excitotoxicity, and has been directly shown to result in neuronal cell loss (Lloyd-Evans et al., 2008, Lloyd-Evans, Pelled et al., 2003a, Pelled, Lloyd-Evans et al., 2003, Pereira, Gazarini et al., 2010, Tessitore, del et al., 2004). The evidence for involvement of Ca^{2+} in the pathophysiology of these diseases is summarised in Table 1 and some of this work has been reviewed previously (Lloyd-Evans et al., 2010). In some cases the main defect has been alterations in Ca^{2+} handling in intracellular stores other than the lysosome (Korkotian, Schwarz et al., 1999), these diseases are not discussed in detail here but information can be found elsewhere (Vitner, Platt et al., 2010). Furthermore, new lysosomal diseases with defects in lysosomal Ca^{2+} homeostasis are being identified. One example being CLN3 disease, a member of the neuronal ceroid lipofuscinoses, caused by mutations in the *CLN3* gene encoding the predominantly lysosomal CLN3 protein of unknown function (Chandrachud, Walker et al., 2015). Cells and tissues from this disease accumulate autofluorescent lipofuscin in lysosomes and have substantial abnormalities in the production and clearance of autophagic vacuoles (Cao, Espinola et al., 2006). A recent study has demonstrated the presence of elevated lysosomal Ca^{2+} in a cerebellar cell line from the CLN3 exon 7/8 1kb deletion mouse model (Chandrachud et al., 2015), the first report of such an increase in any disease. Further work is required to determine the cause of this defect and the role it may play in the pathogenesis of CLN3 disease.

Niemann-Pick disease type C, a disease of reduced lysosomal Ca^{2+} and defective lipid endocytosis

The first reported case of a defect in lysosomal Ca^{2+} in a human disease was that of Niemann-Pick type C (NPC) disease (Lloyd-Evans et al., 2008). NPC is an LSD that manifests primarily with loss of cerebellar Purkinje neurons that results in ataxia, however, neuronal dysfunction is widespread and hallmarks of Alzheimer's have also been reported (Lloyd-Evans & Platt, 2010). Patients usually succumb to the disease in their second decade of life but adult onset forms of the disease as well as milder disease causing mutations have

all been reported (Millat, Marçais et al., 2001, Vanier & Millat, 2003). The disease is caused by mutation in the *NPC1* gene that encodes a 13 transmembrane domain protein of the limiting membrane of the lysosome, also called NPC1 (Lloyd-Evans & Platt, 2010). The exact function of NPC1 remains to be identified but it is believed to be involved in lipid transport, especially cholesterol and potentially sphingolipids and fatty acids (Ioannou, 2005, Lloyd-Evans & Platt, 2010). NPC1 belongs to the eukaryotic family of resistance nodulation division (RND) permease multi-substrate pumps that are capable of transporting antibiotics, heavy metals, detergents, dyes and lipids, all of which have been reported to accumulate in NPC disease lysosomes (Davies, Chen et al., 2000, Kaufmann & Krise, 2008, Tseng, Gratwick et al., 1999). NPC lysosomes accumulate cholesterol, simple sphingolipids including sphingosine, glycosphingolipids, sphingomyelin and lyso-(bis)phosphatidic acid (LBPA) (Lloyd-Evans et al., 2008, Lloyd-Evans & Platt, 2010). In 2008 we determined that multiple cell lines from NPC disease (NPC1 null Chinese hamster ovary cells, NPC1 null mouse astrocytes, NPC patient fibroblasts) as well as cells treated with the NPC1 protein inhibitor U18666a (Lu, Liang et al., 2015) all had reduced levels of lysosomal Ca^{2+} (measured both directly with intra-luminal dextran conjugated Ca^{2+} probes as well as indirect pharmacological release of Ca^{2+} from lysosomes to the cytosol for measurement with a membrane permeant fluorescent Ca^{2+} dye) as well as reduced lysosomal Ca^{2+} release in response to membrane permeant NAADP-AM (Lloyd-Evans et al., 2008). The impact of this defect in lysosomal Ca^{2+} homeostasis in NPC disease is an inhibition of fusion and endocytic transport between the component parts of the endo-lysosomal system, leading to the large accumulation of lipids within NPC cells and tissues (Lloyd-Evans et al., 2008, Visentin, De Nuccio et al., 2013). Indeed the importance of the Ca^{2+} defect to disease pathology was highlighted by the use of curcumin as a potential therapy (Borbon, Hillman et al., 2012, Efthymiou, Steiner et al., 2015, Lloyd-Evans et al., 2008). Curcumin acts to elevate cytosolic Ca^{2+} levels by weak inhibition of SERCA and uncovering of the activity of the ER Ca^{2+} leak channels (Bilmen, Khan et al., 2001). Curcumin led to a significant improvement in function and lifespan of the NPC mouse model (Lloyd-Evans et al., 2008), with a significant rescue of Purkinje cell degeneration in the cerebellum (Smith, Wallom et al., 2009). However any benefit may well have been ameliorated by the action of curcumin against the cytochrome p450 enzymes (Wang, Sun et al., 2015), the activity of which have been shown to be considerably reduced in NPC disease (Nicoli, Al Eisa et al., 2016). The potential benefit of curcumin (in a lipidated vector) has also been questioned (Borbon et al., 2012) as has the cellular benefit (Yu, Swaroop et al., 2014), the low concentrations of curcumin used in this cell based study (2-5 μM) are highly unlikely to induce significant elevation in cytosolic Ca^{2+} in order to rescue endo-lysosomal function in NPC disease cells. Indeed, it is this requirement for higher concentrations of curcumin (10-30 μM (Lloyd-Evans et al., 2008)), higher than those found in the plasma and CSF (0.2-1.8 μM (Mishra & Palanivelu, 2008)), to induce Ca^{2+} elevation within cells that has led to the speculation that curcumin cannot mediate benefit in this way in NPC disease (Borbon et al., 2012). However, it should be noted that its effects are not due to its well-known antioxidant properties (Smith et al., 2009) and low doses of curcumin given to Tg2576 mutant APP overexpressing Alzheimer mice over a period of time were capable of crossing the blood brain barrier and accumulating in the CNS where they stained amyloid deposits (Yang, Lim et al., 2005). As such, it is possible that curcumin eventually reaches a high enough concentration within

cells to weakly inhibit SERCA and induce changes in Ca^{2+} , further work is required in this area.

The defect in lysosomal Ca^{2+} in NPC disease was shown to be triggered by the accumulation of sphingosine (Lloyd-Evans et al., 2008), which has recently been suggested to activate TPC1 and induce emptying of the lysosomal Ca^{2+} store (Hoglinger, Haberkant et al., 2015). This is an interesting hypothesis and one that requires testing by inhibiting the Ca^{2+} release with the NAADP receptor inhibitor Ned19 (Naylor, Arredouani et al., 2009), which should therefore theoretically rescue NPC disease phenotypes. It is also interesting to note that the accumulation of sphingosine in NPC disease cells ($\sim 1\mu\text{M}$ (Lloyd-Evans & Platt, 2010)) is not sufficient to alter lysosomal pH (which is normal in NPC disease (Bach, Chen et al., 1999, Lloyd-Evans et al., 2008)), or induce lysosomal membrane rupture, which can happen at higher concentrations of sphingosine (Kagedal, Zhao et al., 2001, Lloyd-Evans & Platt, 2011). As it has been reported that activation of Ca^{2+} release from lysosomes with NAADP induces a change in lysosomal pH (Morgan & Galione, 2007), it would appear that this enhanced Ca^{2+} release is not occurring constitutively, even though lysosomal Ca^{2+} levels remain constitutively lower (Lloyd-Evans et al., 2008). Furthermore, it has been shown that it requires $\sim 10\mu\text{M}$ sphingosine or more to induce Ca^{2+} release from lysosomes (Lloyd-Evans & Platt, 2011), a concentration ~ 10 fold greater than is actually found in NPC disease storage lysosomes (Lloyd-Evans & Platt, 2010). Another possibility is that sphingosine accumulation is inhibiting the mechanism of Ca^{2+} entry into NPC disease lysosomes, as it is a known inhibitor of Ca^{2+} ATPases (Colina, Cervino et al., 2002, Pandolfi, Schoeffield-Payne et al., 1994), the activity of which have been found on purified lysosomes (Ezaki, Himeno et al., 1992). Clearly more work is required to establish the exact mechanism by which lysosomal Ca^{2+} levels are reduced in NPC disease.

One study has suggested that lysosomal Ca^{2+} levels are in fact not reduced in NPC disease (Shen, Wang et al., 2012). This study is in contention with multiple reports from various research labs (Coen et al., 2012, Hoglinger et al., 2015, Lee, Lee et al., 2010a, Lee, Lee et al., 2014, Lloyd-Evans et al., 2008, Speak, Te Vrugte et al., 2014, Visentin et al., 2013, Xu, Liu et al., 2012). Several of these labs have used either the same NPC patient fibroblast cell line (Visentin et al., 2013, Xu et al., 2012) that was used in the original study (Lloyd-Evans et al., 2008), or a U18666a induced cellular model of NPC disease (Coen et al., 2012, Lloyd-Evans et al., 2008), and all of whom have reported a reduction in lysosomal Ca^{2+} in NPC disease and often by various methods in multiple cells including Purkinje neurons (Lee et al., 2010a). One possibility for the discrepancy in the results is indeed the tools and methods used in the studies. In our initial paper we presented data that NPC disease B lymphoblasts had lower lysosomal Ca^{2+} than controls using the lysosomal cathepsin C substrate GPN alone to burst lysosomes (Lloyd-Evans et al., 2008). The data from the other NPC cell lines used in the paper (CHO, human patient fibroblast and RAW macrophages treated with the NPC1 inhibitor U18666a) was the same but we had to use a slightly different method to fully realise the difference in lysosomal Ca^{2+} that we had also measured with an *in situ* dextran conjugated Ca^{2+} probe (Lloyd-Evans et al., 2008). In these other cell lines we first clamped the other intracellular stores with either thapsigargin (to release ER Ca^{2+}) or ionomycin (to release all intracellular Ca^{2+} stores apart from lysosomes) prior to releasing lysosomal Ca^{2+} with GPN (Lloyd-Evans et al., 2008). We did not require any clamping of the

other intracellular Ca^{2+} stores in the B lymphoblasts as the mechanisms governing potentiation of lysosomal Ca^{2+} release by the ER appear to be different in these immortalised B cells (Waller-Evans and Lloyd-Evans unpublished, (Dellis, Arbabian et al., 2009)). Shown in Fig. 2 is the difference we observe in lysosomal Ca^{2+} content in fibroblasts when we compare cells that have had the intracellular stores clamped prior to either NAADP-AM or GPN addition versus those that have not. As can be seen, there is a substantially smaller lysosomal Ca^{2+} release in the cells which have not been clamped, which correlates more closely with the intra-luminal measurements and the low volumetric content of lysosomes within cells. It is therefore clear that clamping of intracellular stores is crucial in order to conduct accurate indirect measurements of the content of the lysosomal Ca^{2+} store, it is unclear whether this is the case in Shen *et al.*

Is there a role for TRPML1, the lysosomal Ca^{2+} channel defective in mucopolipidosis type IV, in the pathogenesis of Niemann-Pick disease type C?

Inhibition of TRPML1 by lysosomal sphingomyelin has been suggested as a possible mechanism for altering lysosomal Ca^{2+} homeostasis in NPC disease (Shen et al., 2012). That sphingomyelin is unlikely to affect TRPML1 is supported by the findings that inhibition of sphingolipid biosynthesis with myriocin, which reduces sphingosine levels first, rescues the lysosomal Ca^{2+} defect prior to any correction in sphingomyelin levels (Lloyd-Evans et al., 2008). Furthermore, the authors indicate that overexpression of TRPML1 or its activation with the agonist MLSA1 is capable of correcting the defects in endocytic trafficking and cholesterol accumulation observed in different NPC cell lines (Shen et al., 2012). It is therefore interesting to point out that some of their lysosomal Ca^{2+} measurements in NPC cells were made using a construct that overexpresses a Ca^{2+} measuring GCaMP attached to TRPML1 (Shen et al., 2012), which in itself may not distinguish between Ca^{2+} release from lysosomes or more global Ca^{2+} signalling events as highlighted by (Kilpatrick, Yates et al., 2016b). If overexpression of TRPML1 rescues NPC lysosomal phenotypes then theoretically this may correct the defect in lysosomal Ca^{2+} so that there is ultimately no difference to measure. It is also interesting to note that in a separate study by the same group there was no effect of MLSA1 on lysosomal cholesterol in NPC1 null Chinese hamster ovary cells, NPC1 null macrophages or U18666a treated control macrophages (Wang, Gao et al., 2015). This discrepancy is yet to be fully explained. Finally, should TRPML1 be rendered dysfunctional in NPC disease then one would expect some degree of phenotypic overlap between NPC disease cells and cells from the lysosomal disease mucopolipidosis type IV (MLIV) where genetic mutations in the *MCOLN1* gene render the gene product, TRPML1, inactive, dysfunctional or absent (Bach, 2001).

Does pathogenesis of mucopolipidosis type IV mirror Niemann-Pick disease type C?

We have previously summarised the main differences in phenotypes at the cellular level between NPC and MLIV (Lloyd-Evans & Platt, 2011) we now summarise the main differences in patient symptoms (table 2). Although the two diseases are clearly genetically distinct, one would expect some significant degree of overlap in phenotypes if TRPML1 was rendered dysfunctional in both diseases. For example, autofluorescence is well recorded in MLIV cells and tissues but reports of lipofuscin accumulation in NPC disease do not exist in the literature apart from one study (Shen et al., 2012) where apparent autofluorescent

material in NPC cells is co-localised against lysotracker red, a probe well known for its ability to rapidly photoconvert into a green fluorescent molecule (Freundt, Czapiga et al., 2007). At the patient level, a major phenotype in MLIV is elevated blood gastrin levels, iron deficiency in some patients and the presence of achlorhydria from defects in Parietal cell function which affects the acidity of gastric secretions (Schiffmann, Dwyer et al., 1998). There have been no achlorhydria in NPC patients. Ultimately, based on the weight of evidence from a number of different research groups, it would appear that lysosomal Ca^{2+} is reduced in NPC disease. Indeed, this reduced lysosomal Ca^{2+} level defect has been used as a phenotypic output in a high throughput drug screen in NPC patient fibroblasts by the NIH (Xu et al., 2012) and elevation of lysosomal Ca^{2+} has been shown to be beneficial in a number of independent studies (Visentin et al., 2013, Xu et al., 2012).

What is the role of loss of TRPML1 function in the pathogenesis of mucopolidosis type IV?

Loss of function of TRPML1 in MLIV disease results in a clinical phenotype that is embodied by initial rapid neurological decline during early infancy followed by stabilisation for multiple decades (Bach, 2001). The disease is also characterised by ophthalmological defects, abnormal blood gastrin levels and achlorhydria (Schiffmann et al., 1998). At the cellular level, there is an accumulation of lipids (gangliosides and phospholipids (Bach, Zeigler et al., 1980, Bargal & Bach, 1988, Bargal & Bach, 1989)), glycosaminoglycans (Bach, Ziegler et al., 1977) and Fe^{2+} (Dong et al., 2008) within lysosomes that themselves have altered pH and accelerated lipid recycling (Bach et al., 1999, Chen, Bach et al., 1998). Mutations in the *MCOLN1* gene, that encodes TRPML1, are the cause of MLIV (Bargal, Avidan et al., 2000). As mentioned earlier, TRPML1 is a late endosomal and lysosomal ion channel permeant to multiple ions including, Ca^{2+} , H^+ , Na^+ , K^+ and Zn^{2+} (Cuajungco et al., 2014, Dong et al., 2008, LaPlante et al., 2002). Interestingly, recent work has indicated that TRPML1 may be involved in triggering Ca^{2+} release from the ER (Kilpatrick et al., 2016b) suggesting an impact on global Ca^{2+} signalling as well as an ability to regulate autophagy through activation of TFEB via the Ca^{2+} dependent calcineurin (Medina et al., 2015). It has also been reported that TRPML1 is regulated by pH (Cantiello, Montalbetti et al., 2005, Raychowdhury, Gonzalez-Perrett et al., 2004), a finding which was unexpectedly supported in familial Alzheimer's disease where loss of function mutations in *PSEN1* encoding presenilin 1 (PSEN1) leads to defects in lysosomal pH that activate TRPML1 and empty the lysosomal Ca^{2+} store (discussed in more detail below) (Lee et al., 2015). The vast majority of work on TRPML1 has been done using constitutively active mutant forms of TRPML1, how exactly these mutant proteins recapitulate the function of wild-type TRPML1 remains largely unknown (Waller-Evans & Lloyd-Evans, 2015).

Common neurodegenerative diseases with defects in lysosomal Ca^{2+}

Parkinson's disease

Parkinson's disease (PD), one of the most common neurodegenerative diseases, is a progressive idiopathic neurodegenerative disease of ageing that affects >1% of the population aged over 65 and >4% of over 85's (de Rijk, Launer et al., 2000). As well as age, both genetic (*PARK* gene family) and environmental factors (e.g. traumatic brain injuries, exposure to pesticides, exposure to metals such as Mn^{2+}) have been identified as

contributors to the prevalence of this disease (Lai, Marion et al., 2002). PD is the most common movement disorder (Tong, Yamaguchi et al., 2010), the motor defects synonymous with PD are caused by loss of the dopaminergic neurons in the substantia nigra and dopaminergic terminals in the striatum (Dauer & Przedborski, 2003). Loss of neurons is associated with the presence of intracellular inclusions known as Lewy bodies with low molecular weight oligomers of α -synuclein thought to be the primary cause of the neurotoxicity (Planchard, Exley et al., 2014). Evidence that Ca^{2+} signalling is critical in PD for survival of the dopaminergic neurons in the substantia nigra pars compacta comes from the observation of reduced susceptibility to degeneration in neurons expressing the Ca^{2+} binding and buffering protein calbindin-D28K (Yamada, McGeer et al., 1990). Although several studies exist on the role of changes in Ca^{2+} signalling in inducing neuronal excitotoxicity in PD (Schulz, 2007), changes in lysosomal Ca^{2+} signalling have only been explored in recent years. Neuronal cell death in PD is associated to the release of ATP from the cytosol to the extracellular space. This elevation, comparing 1-10nM ATP in the extracellular space to 10mM in the cytosol, results in both an increase in α -synuclein and the activation of ATP responsive P2X channels on neighbouring neurons and the influx of Ca^{2+} into the cell. Interestingly, this elevation in Ca^{2+} mediated by P2X channels was shown to induce lysosomal alkalisation and an increase in autophagic vacuoles (Gan, Moussaud et al., 2015). Further evidence for a potential role of lysosomal Ca^{2+} in PD emerged from a pharmacologically induced model where cathepsin B and L activity, known to be important in lysosomal α -synuclein degradation (McGlinchey & Lee, 2015), was inhibited by Z-Phe-Ala-diazomethylketone. Neurons treated with this inhibitor had expanded lysosomes, elevated lysosomal Ca^{2+} and elevated lysosomal Ca^{2+} release in response to NAADP-AM (Dickinson, Churchill et al., 2010). Similar phenotypes (described below) have now been identified in various PD models.

A subset of the *PARK* genes are associated with lysosomes and the endo-lysosomal system, these include; the lysosomal p-type ATPase 13A2 (*PARK13*), the leucine-rich repeat kinase LRRK2 (*PARK8*) and the lysosomal enzyme glucocerebrosidase (*GBA1*). The *GBA1* gene has recently emerged as a major risk factor for PD with an estimated 10% of sporadic cases of PD thought to be caused by heterozygous mutations in this gene (Sidransky, Nalls et al., 2009). Whilst the exact mechanisms linking reduced activity of glucocerebrosidase (GlcCer) to loss of substantia nigra dopaminergic neurons remain unknown there has been compelling evidence linking mis-folded GlcCer to ER dysfunction. The accumulation of mutant GlcCer in the ER leads to elevated stress (Westbroek, Gustafson et al., 2011) and abnormally elevated ER Ca^{2+} release has been reported in PD patient fibroblasts carrying the N370S mutation in *GBA1* (Kilpatrick et al., 2016a). Indeed, abnormal ER Ca^{2+} release via the ryanodine receptor is a hallmark of Gaucher disease (Korkotian et al., 1999, Pelled, Trajkovic-Bodennec et al., 2005), the most common lysosomal disease caused by loss of function mutations in *GBA1*, and is observed in patient post-mortem brain as well as in mouse neurons where GlcCer is pharmacologically inhibited by conduritol β -epoxide (CBE) (Korkotian et al., 1999, Pelled et al., 2005). In PD caused by heterozygous mutations in *GBA1* the cause of the ER Ca^{2+} defect is likely different to Gaucher disease as it is unlikely that there is any storage of the GlcCer substrate glucosylceramide, as residual enzyme activity must drop to below 15% of normal

for lysosomal glucosylceramide storage to occur as is observed in Gaucher disease (Schueler, Kolter et al., 2004). Further evidence that the ER Ca^{2+} defect in PD does not occur as a result of lipid storage is that this phenotype is observed in PD patient fibroblasts, which are known to not accumulate glucosylceramide (Westbroek, Nguyen et al., 2016). It therefore appears that there is a significant difference between PD caused by heterozygosity in *GBA1* and Gaucher disease as although both diseases have abnormal ER Ca^{2+} homeostasis the underlying cause is different as it is the lipid storage that induces this phenotype in Gaucher disease (Lloyd-Evans et al., 2003a, Lloyd-Evans, Pelled et al., 2003b). It is interesting however to note that a defect in lysosomal Ca^{2+} levels was observed in PD patient fibroblasts carrying the N370S mutation, with lower Ca^{2+} levels being released into the cytosol of the cells following osmotic lysis of lysosomes with GPN (Kilpatrick et al., 2016a). This phenotype appears to resemble NPC disease where the accumulation of Lewy bodies in NPC patient post-mortem brain has been reported (Chiba, Komori et al., 2014), suggesting a potential connection. It is also interesting to note that the activity of GlcCerase is reduced in NPC cells as a result of mis-localisation of the enzyme to late endosomes (Salvioli, Scarpa et al., 2004), alongside the accumulation of cholesterol in both NPC disease and Gaucher disease (Lloyd-Evans & Platt, 2010, Ron & Horowitz, 2008), there are some compelling similarities that warrant further investigation in future. Interestingly, no ER Ca^{2+} defect was observed in fibroblasts where GlcCerase was inhibited by CBE (only in the PD patient fibroblasts heterozygous for *GBA1* mutations, (Kilpatrick et al., 2016a)), which contrasts with earlier studies on Gaucher disease (Korkotian et al., 1999). However it should be noted that although GlcCerase activity was reported as being negligible in these CBE treated cells, the concentration of CBE used (10 μM) was low and based on other studies would potentially inhibit GlcCerase to levels no lower than at least 25% of control (Dermentzaki, Dimitriou et al., 2013), which, based on 15% of enzyme activity being enough to prevent lysosomal storage (Schueler et al., 2004), would not lead to a phenotype resembling Gaucher disease (which would not happen in fibroblasts either as they do not store glucosylceramide) but would in fact be more similar to the level of residual enzyme activity in PD. The very fact that this result with the GlcCerase inhibitor CBE does not resemble what is seen in PD patient cells where GlcCerase is potentially mis-folded again indicates that the mechanisms underlying the Ca^{2+} defects in both diseases are quite different, even if they do ultimately resemble one another.

Autosomal dominant mutations in *PARK8* encoding LRRK2 is one of the most common genetic causes of PD and result in a rare late-onset familial PD that is indistinguishable from sporadic forms of PD (Gomez-Suaga, Luzon-Toro et al., 2012, Tong et al., 2010). Large genome wide association studies (GWAS) have also shown the presence of more common genetic variants in *LRRK2* associated with the risk of non-familial PD (Simon-Sanchez, Schulte et al., 2009). Whilst the exact function of the LRRK2 protein is unknown it has been shown to have both Ras-like GTPase and MAPKKK-like kinase domains (Marin, 2006) and is a protein that is found on the ER as well as endo-lysosomes (Biskup, Moore et al., 2006). A role for LRRK2 in endo-lysosomal function first emerged from the findings that mice null for LRRK2 present with elevated levels of α -synuclein, elevated levels of autophagy, as indicated by an increase in the autophagosomal marker LC3-II, and an increase in lipofuscin, indicative of lysosomal dysfunction (Tong et al., 2010). These findings have been

further supported from research into invertebrate models of PD, the *Drosophila* homologue of LRRK2 (*Lrrk*) localises exclusively to late endosomes and lysosomes where it interacts with and negatively regulates the function of Rab7, a GTPase that regulates endocytic transport. Wild-type *Lrrk* appears to restrict the perinuclear localisation of lysosomes, whilst a *Drosophila* mutant *Lrrk* that resembles the pathogenic human PD causing *LRRK2*^{G2019S} mutation promotes perinuclear lysosomal clustering with the presence of occasionally expanded late endosomes (Dodson, Zhang et al., 2012). Interestingly, expression in mouse primary astrocytes of LRRK2 harbouring not only the common *LRRK2* mutation G2019S but also the expression of other mutations such as R1441C or Y1699C all led to enlarged lysosomes, a phenotype that could be reversed using a LRRK2 kinase inhibitor (Henry, Aghamohammadzadeh et al., 2015). Of interest to the potential role of LRRK2 in modulating endosome and lysosome function in the pathophysiology of PD are the findings from genetic studies of PD patient cohorts indicating increased risk of PD caused by overlap of common variants in *LRRK2* with variants at another PD risk associated locus, *PARK16* (MacLeod, Rhinn et al., 2013, Pihlstrom, Rengmark et al., 2015). Although several genes are contained within this locus, one in particular, namely *RAB7-like variant 1* (*RAB7L1*), provides a further connection to dysfunctional endo-lysosomes in PD and a genetic link between LRRK2 mutations and altered function of endocytic Rab7 GTPases. Furthermore, inhibition of Rab7 in PD patient fibroblasts carrying the common G2019S mutation in *LRRK2* led to a correction in the perinuclear clustering of lysosomes (Hockey, Kilpatrick et al., 2015), providing further evidence of the interplay between LRRK2 and Rab7 in regulating endo-lysosomal function in both healthy cells (negative regulation) and in PD (gain of function). Interestingly, the beneficial effect of inhibiting Rab7 function on lysosomal clustering in PD patient cells could be replicated by genetic silencing of the expression of the lysosomal Ca²⁺ channel TPC2 (but not the endosomal channel TPC1) or inhibition of the TPC2 channel with Ned-19. This benefit was attributed to the presence of a defect in lysosomal Ca²⁺ in the PD patient cells with greater NAADP mediated Ca²⁺ release from lysosomes observed in the PD cells compared to controls (Hockey et al., 2015). Over-expression of TPC2, and elevated lysosomal Ca²⁺ release, has been connected with defects in endocytosis and autophagic vacuole clearance with an associated defect in recruitment of Rab7 to autophagosomes (Lu, Hao et al., 2013). Furthermore, TPC2 has been shown to co-immunoprecipitate with LRRK2 (Gomez-Suaga et al., 2012) raising the possibility that mutant LRRK2 may lead to enhanced activity of TPC2 and greater lysosomal Ca²⁺ release via abnormal TPC2 phosphorylation resulting in lysosomal accumulation and defects in autophagic vacuole clearance via altered Rab7 recruitment. This raises the possibility that treating downstream abnormal lysosomal Ca²⁺ signalling may be a potential therapeutic strategy for certain forms of PD, especially as inhibition of TPC2 with Ned-19 has been shown to rescue the defects in lysosomal clustering (Hockey et al., 2015) and the accumulation of autophagic vacuoles (Gomez-Suaga et al., 2012) associated with abnormal LRRK2 function.

Indeed, the possibility that modulating lysosomal Ca²⁺ is a therapeutic strategy that could be looked at more broadly for PD is supported by the recent study indicating that ambroxol, a drug that is used as an expectorant and is a known chaperone for GlcCerase (Ambrosi, Ghezzi et al., 2015), can induce Ca²⁺ release from lysosomes via alkalinisation (Fois, Hobi et

al., 2015). However, it should be noted that the levels of LAMP1 and 2, LIMP2 and GlcCeramide have all been reported to be lower in multiple regions of the PD brain, as well as the substantia nigra, (Chu, Dodiya et al., 2009, Murphy, Gysbers et al., 2014, Rothaug, Zunke et al., 2014). Although, whether or not these cases of PD were associated with mutations in LRRK2 is unclear but would suggest that further work is required and that the lysosomal Ca^{2+} phenotypes discussed above are not only disparate but also may not be a universal feature of PD.

Alzheimer's disease

Alzheimer's disease (AD) is a complex, multifactorial and polygenic disease of aging that constitutes a major healthcare burden worldwide. AD is a disease of the aging brain where the normal processing, breakdown and recycling of proteins such as the amyloid precursor protein in cells of the brain is deficient. How exactly these defects lead to dysfunction and loss of neurons remains to be fully elucidated but there is an emerging role of lysosomes. For a comprehensive background on the role of lysosomes in AD see (Lloyd-Evans & Haslett, 2016a). As with PD, lysosomal Ca^{2+} defects and an expanded lysosomal system have also been reported in AD (Wilson, Murphy et al., 2004). In particular, FAD caused by mutations in the *PSEN1* gene encoding Presenilin 1 (PSEN1) a component of the γ -secretase complex that cleaves the amyloid precursor protein (Coen et al., 2012). Changes in lysosomal pH have been reported multiple times in PSEN1 null and mutant cells (Avrahami, Farfara et al., 2013, Coffey, Beckel et al., 2014, Lee et al., 2015, Lee, Yu et al., 2010b), suggesting a possible cause of the altered lysosomal Ca^{2+} levels. It has been reported that the lysosomal pH defects in PSEN1 null cells are caused by abnormal glycosylation, in the absence of PSEN1, of the V0a1 subunit of the v-ATPase (Lee et al., 2015, Lee et al., 2010b). This in turn results in reduced v-ATPase activity and an alkalinisation of lysosomes which was proposed to cause defects in lysosomal protein processing and reduced clearance of autophagic vacuoles (Lee et al., 2015, Lee et al., 2010b). It has also been suggested that there is no defect in lysosomal pH in PSEN1 null cells and that the accumulation of autophagic vacuoles and lysosomal processing defects are the result of abnormal fusion between these compartments caused by the reduced lysosomal Ca^{2+} (Coen et al., 2012). Interestingly, these findings suggest a possible mechanistic similarity to NPC disease, discussed above, especially as the NPC1 inhibitor U18666a (Lu et al., 2015) induces the same lysosomal Ca^{2+} dysfunction in all studies (Coen et al., 2012, Lee et al., 2015, Lloyd-Evans et al., 2008). Furthermore, NPC patients present with some hallmarks of Alzheimer like pathology (Saito, Suzuki et al., 2002). However, it appears that the mechanism governing lysosomal Ca^{2+} dysfunction in PSEN1 null cells and NPC disease cells is quite different. The change in lysosomal pH in PSEN1, which is not observed in NPC disease (Bach et al., 1999, Lloyd-Evans et al., 2008), triggers a number of changes in the mechanisms that govern lysosomal Ca^{2+} homeostasis. First, NAADP has been shown to be incapable of dissociating from purified TPC2 when luminal lysosomal pH is less acidic. This results in an inability of NAADP to trigger further Ca^{2+} release via TPC2 (Pitt et al., 2010). We have shown that NAADP-AM signalling is completely inhibited in PSEN1 null cells, providing supportive evidence for a change in lysosomal pH (Lee et al., 2015). This change in NAADP signalling was not caused by any alteration in TPC2 protein levels but could have been the result of overall reduced lysosomal Ca^{2+} levels in the PSEN1 cells. However, NAADP-AM induced

no release from PSEN1 in comparison to U18666a treated cells, an inhibitor of lysosomal NPC1 which induced a similar reduction in lysosomal Ca^{2+} to that seen in PSEN1, where there was ~80% less Ca^{2+} release compared to control (Lee et al., 2015). This suggested to us that TPC2 had been rendered inactive by a change in lysosomal pH. As mentioned above, TRPML1 has been suggested to be activated following a change in lysosomal pH, potentially operating in late endosomes or homotypic organelles (Piper & Luzio, 2004, Raychowdhury et al., 2004). Based on the change in lysosomal pH in PSEN1 cells we tested this theory and found that the synthetic agonist of TRPML1, MLSA1, was able to induce significantly more Ca^{2+} release from PSEN1 lysosomes (Lee et al., 2015). We concluded that hyperactive TRPML1 was the cause of the alteration in lysosomal Ca^{2+} as inhibition of PIKfyve, the enzyme that creates $\text{PI}(3,5)\text{P}_2$ the endogenous ligand of TRPML1, was able to normalise PSEN1 lysosomal Ca^{2+} (Lee et al., 2015). It is also interesting to note that the amyloid precursor protein (APP) itself binds to PIKfyve and regulates the levels of $\text{PI}(3,5)\text{P}_2$ (Currinn, Guscott et al., 2016) providing the possibility that APP itself regulates TRPML1 activity.

Interestingly, TRPML1 activity has also been shown to be altered in other forms of dementia. Amyloid- β accumulates by an unknown mechanism within neurons of brains infected with HIV and have been shown to accumulate in lysosomes laden with Ca^{2+} and sphingomyelin (Bae, Patel et al., 2014). Interestingly, activation of TRPML1 cleared both the amyloid accumulation as well as the accumulation of sphingomyelin in cultured neurons expressing the HIV coat protein gp120. Although this is the opposite of what we found in FAD, the cellular phenotypes in part resemble NPC disease, where there is also an accumulation of sphingomyelin. Furthermore, NPC1, sometimes referred to as a form of childhood dementia owing to the earliest recorded presence of neurofibrillary tangles, hyperphosphorylated tau and accumulation of amyloid- β_{40-42} in post-mortem tissue samples from young patients (Saito et al., 2002, Suzuki, Parker et al., 1995), also has altered Ca^{2+} release from TRPML1 (Shen et al., 2012). This is presumably in response to reduced late endosome/lysosomal Ca^{2+} levels rather than either a change in lysosomal pH (not present in NPC), or an effect of sphingomyelin on TRPML1 function as discussed above. Whether or not the reduced activity of TRPML1 in NPC disease is in any way involved with the cellular hallmarks of AD remains to be elucidated but there is clearly a role for altered lysosomal Ca^{2+} in certain forms of AD, demonstrating whether this feature also occurs in sporadic AD may be crucial for the development of any therapeutic strategy in this area.

To conclude, changes in lysosomal Ca^{2+} are becoming increasingly prevalent in various forms of both common and rare forms of neurodegenerative disease. The development of clinically approved therapeutics in the future that can target and alter lysosomal Ca^{2+} signalling may be an important avenue for ultimately treating these diseases.

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Extracellular fluid (plasma, interstitial fluid, cerebrospinal fluid)
pH= ~7 - 7.4, Ca^{2+} = 1.2 - 1.8mM

Figure 1. Maturation of the endocytic system is defined by the indicated changes in pH and Ca^{2+} concentration as well as the expression of ion channels.

Extracellular Ca^{2+} levels is high, yet the process of acidification by the vacuolar ATPase (vATPase) results in Ca^{2+} release out of early endosomes which drives vesicular transport to late endosomes via Ca^{2+} dependent protein machinery such as Annexin A2. Late endosomes and lysosomes have lower pH and higher Ca^{2+} levels which are necessary both for localised events such as vesicular recycling and fusion between late endosomes and lysosomes but also for triggering global changes in Ca^{2+} via triggering of ER Ca^{2+} release. Several new ion channels (indicated and discussed in the text) have been identified on the lysosomal

membrane in recent years, highlighting the importance of this Ca^{2+} store to the cell. Green arrows indicate direction of endocytosed cargo (grey circles with black border represent transport vesicles), red arrow indicates fusion between late endosomes and lysosomes, all of these are processes that require local Ca^{2+} release.

Figure 2. Lysosomes induce Ca^{2+} release from the ER.

Utilisation of either GPN to burst lysosomes to measure Ca^{2+} levels or NAADP-AM to release Ca^{2+} from lysosomes results in a large amount of Ca^{2+} release (Fig Ai and Aii). This is the result of lysosomal Ca^{2+} release inducing further Ca^{2+} release from the ER (Fig Bi) which can mask real changes in lysosomal Ca^{2+} levels in cells. In order to properly quantify lysosomal Ca^{2+} it is necessary to clamp the Ca^{2+} release from the other stores, this can be done by pretreatment of the cells with an inhibitor of Ca^{2+} mobilisation such as thapsigargin which blocks Ca^{2+} uptake into the ER via inhibition of SERCA that subsequently uncovers Ca^{2+} leak out of the ER (Ai and Aii). Following release of the ER store, GPN or NAADP-AM can be added to give a more accurate, smaller, estimate of lysosomal Ca^{2+} , this is summarised in Bii.

Intracellular Ca²⁺ defects associated with the lysosomal storage diseases.

Table 1

Disease	Disease cause and phenotypes	Description of Ca ²⁺ defect	Cause of the Ca ²⁺ defect	Functional consequence of disrupted Ca ²⁺
<i>Lysosomal Ca²⁺ defects</i>				
Niemann-pick type C	Mutations in the NPC1 gene encoding a lysosomal transmembrane protein believed to be involved in lipid transport. Patient cells accumulate multiple lipids within lysosomes including cholesterol, sphingomyelin, sphingosine and glycosphingolipids	Reduced intravesicular levels resulting in reduced Ca ²⁺ release via TPC2 and potentially TRPML1	Lysosomal accumulation of sphingosine	Disrupted endocytosis and lysosomal lipid storage, pharmacological elevation of Ca ²⁺ rescues Purkinje neuron death in the mouse model
Mucopolidiosis type IV	Mutations in the MCOLN1 gene encoding a late endosomal and lysosomal ion channel permeant to Ca ²⁺ , Fe ²⁺ , Na ⁺ , K ⁺ and Zr ²⁺ . Patient cells are characterised by intralysosomal accumulation of phospholipids, sphingoids, mucopolysaccharides and free Fe ²⁺	Potential elevation in lysosomal Ca ²⁺ content	Loss of function in late endosomal/lysosomal Ca ²⁺ channel TRPML1	Defects in clearance of autophagic vacuoles within neurons
CLN3 disease	Mutations in the CLN3 gene encoding a lysosomal transmembrane protein, CLN3, of unknown function. cells accumulate autofluorescent lipofuscin	Elevated lysosomal Ca ²⁺	Currently unknown	Dirupted clearance of autophagic vacuoles
Mucopolysaccharidosis type I	Mutation of the IDUA gene encoding a-L-iduronidase which is a lysosomal enzyme that cleaves glycosaminoglycans, which accumulate in MPS type I	Elevated lysosomal Ca ²⁺	Attributed to lysosomal deacidification	Elevated levels of apoptosis
<i>ER Ca²⁺ effects</i>				
Gaucher disease	Mutation in GBA1 encoding acid b-glucosidase orglutosyl/ceramidase. Cells accumulate the enzyme substrate, the glycosphingolipid glueosyl/ceramide within lysosomes	Enhanced Ca ²⁺ release from the ER via potentiation of ryanodine receptors	Accumulating glucosylceramide, the lysosomal storage substance in Gaucher disease affecting ER function	Neuronal excitotoxicity and death as a result of elevated cytosolic Ca ²⁺
GM1 gangliosidosis	Mutation in the GLB1 gene encoding lysosomal b-galactosidase. Cells accumulate the enzyme substrate ganglioside GM1, a important neuronal glycosphingolipd, in lysosomes	Inhibition of ER Ca ²⁺ entry via SERCA, elevated mitochondrial Ca ²⁺	Accumulating ganglioside GM1, the Disrupted mitochondrial function. ER stress substrate of the lysosomal b-gafactosWase and presence of the unfolded protein enzyme defective in GM1 gangliosidosis, response, altering ER function	Disrupted mitochondrial function, ER stress and presence of the unfolded protein response
GM2 gangliosidosis (Saneshoff disease)	Mutation in HEXB gene encoding lysosomal b-hexosaminidase which degrades the intermediate ganglioside GM2 in lysosomes and which accumulates in Sandhoff disease	Inhibition of ER Ca ²⁺ entry via SERCA	Accumulating ganglioside GM2, the substrate of the lysosomal b-hexosaminidase enzyme defective in GM2 gangliosidosis, altering ER function	Enhanced sensitivity of primary neurons to excitotoxicity
Niemann-Pick disease type A	Mutation in the SMPD1 gene encoding the lysosomal enzyme acid sphingomyelinase, responsible for the degradation of the phospho-sphingolipd sphingomyelin within lysosomes	Inhibition of plasma membrane and ER Ca ²⁺ ATPase activity reuslting in elevated cytosolic Ca ²⁺	The accumulating lipid substrate of acid sphingomyelinase, the enzyme defective in Niemann-Pick A, sphingomyelin is implicated but mechanism of inhibition currently unknown	Elevated oxidate stress in neurons from mouse model of NPA and from a NPA patient
LSDs with other Ca ²⁺ defects Krabbe disease				
Fabry disease	Mutation in the GLA gene encoding for a-galactosidase A. Loss of function causes the enzyme substrate, globoside, a glycosphingolipd, to accumulate	Enhanced voltage gated Ca ²⁺ currents and elevated Ca ²⁺ entry into sensory neurons	Lyso-globoside, a storage molecule in Fabry disease acts on plasma membrane voltage dependent Ca ²⁺ channels	Potential cause of peripheral neuropathy
Pompe disease	Mutation in the GAA gene enoding a-glucosidase which breaks down glycogen within lysosomes	Mitochondrial Ca ²⁺ overload and up-regulation of the Blsubunit of the L-type Ca ²⁺ channel	Potential premature ageing of cells	Compromised mitochondrial respiration in whole muscle fibers
<i>LSDS without Ca²⁺ defects</i>				

Disease	Disease cause and phenotypes	Description of Ca ²⁺ defect	Cause of the Ca ²⁺ defect	Functional consequence of disrupted Ca ²⁺
Cystinosis	Mutation in the CTN5 gene that encodes cystinosin lysosomal transmembrane protein involved in the efflux of L-cystine from lysosomes	No lysosomal Ca ²⁺	N/A	N/A

Notes: Niemann-Pick C, mucopolipidosis type IV and CLN3 disease are reviewed in this article, mucopolysaccharidosis type I (Pereira et al., 2010), the lysosomal diseases with associated ER Ca²⁺ signalling defects (Ginzburg and Futerman, 2005; Korkotian et al., 1999; Pelled et al., 2003; Tessitore et al., 2004), Krabbe (Im, et al., 2001), Fabry (Choi, et al., 2015), Pompe (Lim, et al., 2015), and cystinosis (Ivanova, et al., 2016) are discussed elsewhere as indicated.

Table 2
Clinical differences and similarities between Niemann-Pick type C and mucopolipidosis type IV patients.

	Mucopolipidosis type IV	Niemann-Pick type C
Neurological symptoms		
Cerebellar neuron loss	Present late in disease progression	Early event in pathology
Thinned corpus callosum	Present	Not Present
Developmental delay	Severe	Present
Cognitive impairment	Present	Present
Epilepsy	Not present	Present
Cataplexy	Not present	Present
Dystonia	Not present	Present
Tremor	Not present	Present
Psychiatric disorders	Not present	Present
Dementia	Not present	Present
Hearing loss	Not present	Present
Ophthalmic symptoms		
Ocular-motor abnormalities	Not present	Present
Corneal clouding	Present	Not present
Retinal degeneration	Present	Not present
Strabismus	Present	Not present
Visceral symptoms		
Hepatosplenomegaly	Not present	Present
Cholestasis	Not present	Present
Dysphagia	Present	Present
Dysarthria	Present	Present
Achylorria	Present	Not present
Elevated gastrin	Present	Not present
Poor physical growth	Present	Not present
Hypotonia	Present	Present
Lung disease	Not present	Present
Disease progression		
Progressive decline	Not present	Present
Stability after initial decline	Present	Not present

Notes: Overall, only 4/26 phenotypes are shared and these are features that are aligned amongst most neurodegenerative diseases (Altarescu, Sun et al., 2002; Patterson, et al., 2012; Patterson, et al., 2013; Schiffmann et al., 1998; 2014; Smith, et al., 2002). It appears there is little similarity between NPC and MLIV at the patient level, as is the case at the cellular level (Lloyd-Evans and Platt, Cell Calcium 2011).