

**Lipid-mediated motor-adaptor sequestration impairs
axonal lysosome delivery leading to autophagic
stress and dystrophy in Niemann-Pick type C**

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Abbreviations: AV: autophagic vacuole; AVd: degradative autophagic vacuole; AVi: initial autophagic vacuole; CBD: cargo-binding domain; DIV: days *in vitro*; DRG: dorsal root ganglion; GCase: glucocerebrosidase; HPCD: 2-hydroxypropyl- β -cyclodextrin; KHC: kinesin heavy chain; KIF5: kinesin-1 family; LAMP1: lysosome associated membrane protein 1; LSD: lysosomal storage disorder; MD: motor domain; NPC: Niemann-Pick disease type C; SKIP: SifA and kinesin-interacting protein; STED: stimulated emission and depletion; TEM: transmission electron microscopy; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling; WT: wild type.

SUMMARY

Niemann-Pick disease type C (NPC) is a neurodegenerative lysosomal storage disorder characterized by lipid accumulation in endolysosomes. An early pathologic hallmark is axonal dystrophy occurring at presymptomatic stages in NPC mice. However, the mechanisms underlying this pathologic change remain obscure. Here, we demonstrate that endocytic-autophagic organelles accumulate in NPC dystrophic axons. Using super-resolution and live-neuron imaging, we reveal that elevated cholesterol on NPC lysosome membranes sequesters kinesin-1 and Arl8 independent of SKIP and Arl8-GTPase activity, resulting in impaired lysosome transport into axons, contributing to axonal autophagosome accumulation. Pharmacologic reduction of lysosomal membrane cholesterol with 2-hydroxypropyl- β -cyclodextrin (HPCD) or elevated Arl8b expression rescues lysosome transport, thereby reducing axonal autophagic stress and neuron death in NPC. These findings demonstrate a pathological mechanism by which altered membrane lipid composition impairs lysosome delivery into axons and provide biological insights into the translational application of HPCD in restoring axonal homeostasis at early stages of NPC disease.

INTRODUCTION

Lysosomes play key roles in maintaining cellular homeostasis by degrading and recycling substrates received from the endocytic and autophagic pathways (Klionsky and Emr, 2000; Levine and Klionsky, 2004; Mellman, 1996). Degradation of endocytic and autophagic cargos depends on their dynamic interactions with lysosomes, which provide the acid hydrolases required for substrate degradation (Luzio et al., 2007). To carry out this function efficiently, lysosomes must move bidirectionally along microtubules (MTs) throughout the cytoplasm (Bonifacino and Neefjes, 2017; Pu et al., 2016). However, this process is particularly challenging in neurons because of their highly polarized morphology with axons that can extend up to 1 meter in length. Neurons are therefore highly vulnerable to various defects in lysosome trafficking, positioning, and function, which are associated with both rare and common neurodegenerative diseases (Ferguson, 2019; Marques and Saftig, 2019; Platt et al., 2018; Sharma et al., 2018). While enzymatically active lysosomes are enriched in the cell body (Cai et al., 2010; Gowrishankar et al., 2017; Gowrishankar et al., 2015; Yap et al., 2018), mature lysosomes and lysosome-like organelles are also found in distal axons (Farfel-Becker et al., 2019; Farias et al., 2017; Gowrishankar et al., 2017; Jin et al., 2018; Lee et al., 2011). However, the impact of lysosome anterograde trafficking and positioning on axonal pathology in neurodegenerative diseases remains largely elusive.

Niemann-Pick disease type C (NPC) is a neurodegenerative lysosomal storage disorder (LSD) characterized by the accumulation of multiple lipids in the endolysosomal compartment (Lloyd-Evans et al., 2008). Loss-of-function mutations in the lysosomal membrane protein NPC1 account for approximately 95% of NPC cases (Carstea et al., 1997), while the other 5% are caused by mutations in the lysosomal luminal protein NPC2 (Naureckiene et al., 2000), though the two forms are clinically indistinguishable (Mengel et al., 2013; Vanier, 2010). One major pathologic feature observed in NPC patients and recapitulated in mouse models of NPC disease is axonal dystrophy, which consists of bulbous swellings containing accumulated organelles (Boland and Platt, 2015; Walkley, 1998; Walkley et al., 2010; Walkley and Suzuki, 2004). These axonal changes precede symptom onset and neurodegeneration in NPC mice, suggesting that defects in the trafficking and degradation of these axonal organelles contribute to early NPC pathology. These clinical implications highlight the importance of understanding the cellular events that precede axonal organelle accumulation and their contribution to neurodegeneration. However, the mechanisms underlying these early pathologic events in NPC neurons remain obscure.

In this study, we reveal a lipid-linked pathological mechanism underlying autophagic stress and organelle accumulation in NPC dystrophic axons. Using live-neuron imaging combined with genetic and pharmacologic interventions, we demonstrate that chronic lysosomal dysfunction in NPC neurons compromises the axonal trafficking and positioning of mature degradative lysosomes, which impairs autophagy-lysosomal clearance, leading to the accumulation of autophagic organelles within the axon. Axonal lysosome delivery is driven by the kinesin-1-SifA and kinesin-interacting protein (SKIP)-Arl8 complex, where the small GTPase Arl8b links lysosomes to MT-based kinesin-1 motors through its effector SKIP (Farias et al., 2017). Our findings demonstrate a lipid-mediated disruption to the assembly of the kinesin-1-SKIP-Arl8 transport complex on NPC lysosomes. Abnormal cholesterol accumulation on lysosome membranes contributes to impaired lysosome trafficking and positioning in distal axons of NPC neurons, leading to reduced axonal degradation capacity, thereby contributing to axonal dystrophy and degeneration. Our study highlights the importance of soma-derived axonal lysosomes in the maintenance of axonal homeostasis and reveals a mechanism underlying their defective axonal delivery in NPC. This study also provides biological insights into the translational application of 2-hydroxypropyl- β -cyclodextrin (HPCD) in reducing lysosomal membrane cholesterol and restoring axonal degradation capacity in the early stages of NPC disease.

RESULTS

Axonal Autophagic Stress in *Npc1*^{-/-} Mice Starting at Presymptomatic Stages

To characterize the organelle accumulations within *Npc1*^{-/-} dystrophic axons, we first examined their composition in dorsal root and cortical axons from symptomatic *Npc1*^{-/-} mice corresponding to postnatal day 50-60 (P50-60), representing two regions vulnerable to neurodegeneration (Ohara et al., 2004; Pressey et al., 2012). Using transmission electron microscopy (TEM), we observed a striking buildup of endocytic and autophagic organelles within dystrophic axons consisting primarily of autophagic vacuoles (AVs), multivesicular bodies, and multilamellar vesicles (**Figures 1A and 1B**). Notably, the majority of AVs in *Npc1*^{-/-} swollen axons exhibited features of early stage initial AVs (AVi) characterized by a double-membrane bilayer containing electron-lucent material, while fewer late AVs in the degradative stage (AVd) following fusion with lysosomes (Klionsky et al., 2021) were observed. Importantly, these axonal phenotypes were not found in age-matched wild type (WT) mice. Such striking accumulations represent intermediates of the endo-lysosomal-autophagic pathways, indicating defects in their trafficking, maturation, and degradation in *Npc1*^{-/-} distal axons.

We next aimed to understand the early dynamic events that might underlie AV accumulation by assessing their distribution and trafficking in neuronal axons from presymptomatic *Npc1*^{-/-} mice corresponding to P30-40. We used dorsal root ganglion (DRG) neurons because (1) they display *in vivo* axonal organelle accumulation, (2) represent a rigorous model amenable to culture from adult mice in our previous studies (Cheng et al., 2015; Farfel-Becker et al., 2019; Lin et al., 2017; Xie et al., 2015), and (3) almost all neurites are axons with uniformly polarized MTs (Maday and Holzbaur, 2012) to allow for directional analyses of organelle transport along axons. DRG neurons isolated from P30-40 mice were nucleofected with AV marker EGFP-LC3 at 0 days *in vitro* (DIV0), followed by live imaging at DIV3-4. Only distal axons with uniform diameter were selected for live imaging. In WT axons, EGFP-LC3 appeared mostly diffuse as cytosolic LC3I (**Figure 1C**). However, in *Npc1*^{-/-} axons, EGFP-LC3 was more punctate as lipidated LC3II incorporated into AV membranes. Strikingly, *Npc1*^{-/-} axons showed a robust increase in AV density compared with control (WT: 1.02 ± 0.18 ; *Npc1*^{-/-}: 9.21 ± 0.69 ; $p < 0.0001$; per 100- μ m axon length; **Figure 1D**), reflecting axonal autophagic stress, consistent with our TEM observations in adult *Npc1*^{-/-} mice.

Because most axonal autophagosomes are generated distally and exhibit predominant retrograde transport towards the soma for degradation (Cheng et al., 2015; Lee et al., 2011; Maday et al.,

2012), we hypothesized that AV accumulation in *Npc1*^{-/-} distal axons may reflect defects in their retrograde motility. To test this, we monitored axonal AV trafficking in DRG neurons cultured from P30-40 mice. In WT neurons, EGFP-LC3-labeled AVs moved predominantly ($57.63 \pm 7.35\%$) in the retrograde direction (**Figures 1E and 1F, Videos S1, S2**), consistent with previous studies (Cheng et al., 2015; Khobreakar et al., 2020; Kimura et al., 2008; Lee et al., 2011; Maday et al., 2012). However, contrary to our hypothesis, axonal AVs in *Npc1*^{-/-} neurons displayed similar motility patterns to WT neurons, suggesting that increased AV density in *Npc1*^{-/-} axons does not result from defects in their retrograde transport. Since axonal AV maturation is linked to their retrograde motility, this prompted us to determine their maturation using the mRFP-EGFP-tagged LC3 reporter (Kimura et al., 2007). AVs in distal axons of *Npc1*^{-/-} neurons showed a higher percentage of immature AVs co-labeled by both mRFP and EGFP compared with WT (**Figure S1**). In contrast, the majority of AVs in WT axons displayed a mature status (only retaining mRFP signal) under our conditions. These findings indicate a defective capacity of axonal AVs to achieve maturation and degradation during their retrograde trafficking in presymptomatic *Npc1*^{-/-} axons.

Reduced Lysosome Positioning in Distal Axons from Presymptomatic *Npc1*^{-/-} Mice

Mature lysosomes provide the active hydrolases for degradation of endocytic and autophagic cargos. Since the majority of AVs in *Npc1*^{-/-} dystrophic axons represent immature AVi-like structures (**Figures 1A and 1B**), we next asked whether axonal lysosome distribution is altered in DRG neurons from presymptomatic (P30-40) *Npc1*^{-/-} mice. Given that the lysosome associated membrane protein 1 (LAMP1) is distributed among endo-lysosomal and autophagic organelles (Cheng et al., 2018), we used LAMP1 combined with activity-based lysosome probes to monitor degradative lysosomes. We first examined axonal lysosome distribution using MDW933, a fluorescent probe that binds specifically to the active form of the lysosomal hydrolase glucocerebrosidase (GCase) under acidic conditions (Westbroek et al., 2016; Witte et al., 2010). Strikingly, *Npc1*^{-/-} neurons displayed an approximately six-fold decrease in the number of active lysosomes positioned along axons relative to WT neurons (WT: 15.02 ± 1.50 ; *Npc1*^{-/-}: 2.27 ± 0.48 ; $p < 0.0001$; per 100- μm axon length; **Figures 2A and 2B**), representing reduced degradative capacity in *Npc1*^{-/-} distal axons. Since activity-based lysosome probes label enzymatically active hydrolases, we next tested whether this reduction resulted from impaired lysosome positioning in distal axons by examining LAMP1-labeled endolysosomes. Notably, *Npc1*^{-/-} axons displayed a two-fold reduction in the number of LAMP1-labeled organelles compared with WT (WT: 17.75 ± 1.37 ; *Npc1*^{-/-}: 8.41 ± 1.35 ; $p < 0.0001$; per 100- μm axon length; **Figures 2C and 2D**), reflecting decreased endolysosome density in *Npc1*^{-/-} DRG axons at presymptomatic stages.

We next determined whether decreased lysosome density along axons could also be detected in central nervous system (CNS) neurons. To address this, we examined embryonic cortical neurons cultured in microfluidic devices that physically and fluidically separate axons from cell bodies and dendrites by an array of 450- μ m-long microgrooves (Taylor et al., 2005). Neurons are plated in the soma/dendritic chamber and by DIV5, only axons that enter the microgrooves extend far enough to grow into the axon chamber, thus allowing analysis of lysosomes in distal axons. *Npc1*^{-/-} cortical axons displayed a two-fold reduction in active GCase-labeled lysosomes (WT: 13.49 ± 1.0 ; *Npc1*^{-/-}: 6.32 ± 0.57 ; $p < 0.0001$; per 100- μ m axon length; **Figures 2E and 2F**) and LAMP1-labeled endolysosomes compared with WT (WT: 18.18 ± 1.34 ; *Npc1*^{-/-}: 8.64 ± 0.87 ; $p < 0.0001$; per 100- μ m axon length; **Figures 2G and 2H**), indicating that reduced axonal lysosome density occurs at early stages of disease progression in embryonic CNS neurons.

We next tested whether such a robust reduction in axonal lysosome density in *Npc1*^{-/-} cortical neurons reflected an overall decrease in neuronal lysosomes. Notably, *Npc1*^{-/-} neurons at DIV8 exhibited increased integrated density of active GCase ($p < 0.0001$), LAMP1 ($p < 0.0001$), and active cathepsin D ($p < 0.0001$) in the cell body relative to WT (**Figures 2I-2L**). Total protein levels of GCase, LAMP1, and cathepsins B and D in DIV7 cortical neurons also displayed no reduction in *Npc1*^{-/-} compared with WT (**Figures 2M and 2N**). We also measured mRNA levels of several TFEB targets (Sardiello et al., 2009), and observed increased *Ctsd* mRNA levels but no significant changes in the transcript levels of the other TFEB targets *Ctsb*, *Lamp1*, *Atp6v0e1*, or *Atp6v1h* (**Figure S2A**), suggesting that the increase in *Ctsd* mRNA may not represent a TFEB-mediated response. In addition, filipin staining to detect unesterified cholesterol showed that *Npc1*^{-/-} neurons store substantial levels of cholesterol in the soma compared with WT (**Figures S2B and S2C**), consistent with a cellular phenotype of NPC. Taken together, these results suggest that the lower number of degradative lysosomes in *Npc1*^{-/-} axons and their increased accumulation in the soma may reflect impaired anterograde trafficking and/or positioning of lysosomes into axons.

Impaired Anterograde Transport of Degradative Lysosomes into *Npc1*^{-/-} Axons

Our recent study revealed that soma-derived active lysosomes are continuously delivered to axons to maintain distal degradation capacity (Farfel-Becker et al., 2019). Increased lysosome accumulation in cell bodies and reduced lysosome density in distal axons prompted us to ask whether somatic lysosomes were not being delivered properly to distal axons in *Npc1*^{-/-} neurons. To address this, we loaded active GCase probe MDW941 or active cathepsin D probe BODIPY-

FL-pepstatin A into the soma/dendritic chamber of a microfluidic device for 30 min, followed by live imaging the axon chamber for a total period of 90 min (**Figure 3A**). Our previous study confirmed the fluidic restriction of activity-based lysosome probes loaded in the soma/dendritic chamber, and demonstrated that their dynamic influx into the axon chamber represents MT-based anterograde lysosome transport (Farfel-Becker et al., 2019). After soma/dendritic chamber loading with MDW941 (**Figures 3B and 3C**) or BODIPY-FL-pepstatin A (**Figures 3D and 3E**), we observed degradative lysosomes delivered to distal axons from the cell body. Strikingly, *Npc1*^{-/-} neurons displayed a progressive reduction in the axonal delivery of active lysosomes relative to WT at DIV7 (MDW941: $p < 0.0001$; BODIPY-FL-pepstatin A: $p = 0.0015$) and further at DIV10 (MDW941: $p < 0.0001$; BODIPY-FL-pepstatin A: $p < 0.0001$).

To determine whether this defect in axonal lysosome delivery represents a specific impairment in anterograde transport, we next monitored lysosome motility in distal axons. In WT axons, active GCa6-labeled degradative lysosomes exhibited bidirectional transport with motile lysosomes accounting for approximately 60% of the total axonal lysosome pool (**Figures 3F and 3G, Videos S3, S4**); of which $36.29 \pm 2.41\%$ moved in the retrograde direction, and $23.93 \pm 2.74\%$ moved anterogradely towards the distal terminal. However, active GCa6-labeled lysosomes in *Npc1*^{-/-} distal axons displayed reduced anterograde transport ($12.59 \pm 2\%$; $p = 0.0022$) with no significant changes in stationary or retrograde motility. We also monitored lysosome motility in distal axons using BODIPY-FL-pepstatin A. Consistently, *Npc1*^{-/-} neurons showed impaired anterograde transport of lysosomes containing active cathepsin D (WT: $33.22 \pm 2\%$; *Npc1*^{-/-}: $21.6 \pm 3.36\%$; $p = 0.0038$) but with a corresponding increase in stationary puncta (WT: $27.92 \pm 2.22\%$; *Npc1*^{-/-}: $45.23 \pm 3.56\%$; $p < 0.0001$) and no significant change in retrograde motility (**Figures 3H and 3I**). Lysosomes in *Npc1*^{-/-} axons displayed reduced flux rates without significant changes in their speed or velocity (**Figure 3J**). We also observed no changes in axonal mitochondrial density or motility in *Npc1*^{-/-} neurons compared with WT (**Figures S3A-S3D**). These results support the notion that impaired lysosome transport into *Npc1*^{-/-} axons does not represent a global defect in axonal organelle transport.

This led us to ask whether decreased lysosome density along axons impacts axonal autophagic clearance in *Npc1*^{-/-} neurons. While a robust AV increase was detected in DRG axons from presymptomatic (P30-40) *Npc1*^{-/-} mice (**Figures 1C and 1D**), it is not known if embryonic *Npc1*^{-/-} cortical neurons also display axonal autophagic stress. To address this, neurons cultured in microfluidic devices were transduced with EGFP-LC3 at DIV0, followed by live imaging at DIV8

and DIV10. We found no significant difference in the density of AVs in *Npc1*^{-/-} axons at DIV8 (**Figures S3E and S3F**), a time point where lysosome density was already reduced (**Figures 2E-2H**). However, at DIV10, AV density was significantly increased in *Npc1*^{-/-} axons (WT: 2.86 ± 0.44 ; *Npc1*^{-/-}: 4.76 ± 0.62 ; $p = 0.0122$; per 100- μ m axon length). Notably, this increase in axonal AV density did not reflect changes in their transport as no differences in their motility were observed (**Figures S3G and S3H, Videos S5, S6**). These results indicate that lysosome transport defects precede axonal AV accumulation in *Npc1*^{-/-} cortical neurons, suggesting that impaired axonal lysosome delivery may contribute to autophagic stress in *Npc1*^{-/-} axons.

Aberrant Sequestration of Kinesin-1 and Arl8 on *Npc1*^{-/-} Lysosome Membranes

These findings prompted us to examine NPC-linked mechanisms underlying impaired anterograde axonal lysosome transport. A previous study established that the kinesin-1-SKIP-Arl8 complex drives lysosome transport into axons (Farias et al., 2017). Therefore, we hypothesized that this complex might be improperly recruited to *Npc1*^{-/-} neuronal lysosomes, and thus underlie impaired lysosome delivery into *Npc1*^{-/-} axons. To test this, we applied STED super-resolution microscopy to examine the localization of kinesin-1, SKIP, and Arl8 on somatic lysosomes of cortical neurons. Unexpectedly, we observed a striking phenotype: while both endogenous kinesin-1 and Arl8 were largely dispersed throughout the soma of WT neurons, they appeared accumulated on the surface of LAMP1-labeled endolysosomes in *Npc1*^{-/-} neurons (**Figures 4A-4D**). In contrast, SKIP did not show similarly enhanced association with *Npc1*^{-/-} lysosome membranes (**Figures 4E and 4F**). These super-resolution images suggest that kinesin-1 is not recruited to *Npc1*^{-/-} neuronal lysosomes by the GTPase effector SKIP and therefore may represent a non-functional sequestration of kinesin-1 and Arl8 on *Npc1*^{-/-} lysosome membranes.

Kinesin-1 family members (known as KIF5) drive the anterograde transport of lysosomes (Farias et al., 2017; Palomo-Guerrero et al., 2019). Each KIF5 heavy chain (KHC) contains an N-terminal motor domain (MD) and a C-terminal cargo-binding domain (CBD) that mediates an association with kinesin light chains or directly interacts with a cargo adaptor (Hirokawa et al., 2010). To test whether KHC may be sequestered non-specifically, we constructed two truncated mutants of KIF5B KHC: EGFP-tagged KHC-MD and KHC-CBD. STED imaging revealed that while both KHC-MD and KHC-CBD were largely dispersed throughout WT neurons, their enrichment was detected on the surface of lysosomes in *Npc1*^{-/-} neurons (**Figures 4G-4J**), suggesting the non-specific sequestration of KHC independent of cargo binding.

We further tested whether kinesin-1 sequestration depends on the normal targeting pathway by knockdown of Arl8 or SKIP (**Figures S4A-S4D**). STED imaging showed no change in the percentage of LAMP1-labeled areas associated with kinesin-1 signals following depletion of Arl8 ($p = 0.7437$; **Figures 5A and 5B**) or SKIP ($p > 0.99$; **Figures 5C and 5D**), suggesting that kinesin-1 sequestration occurs independent of the Arl8-SKIP targeting pathway. We next determined whether the sequestration of kinesin-1 and Arl8 is affected by Arl8 GTPase activity. We expressed WT, GTP-locked mutant Q75L (constitutively active), or GDP-locked mutant T34N (constitutively inactive) (Hofmann and Munro, 2006) forms of Arl8b-mCh in *Npc1*^{-/-} cortical neurons at DIV4, followed by immunostaining at DIV8. We found that (1) accumulation of kinesin-1 on *Npc1*^{-/-} lysosome membranes is independent of Arl8 GTPase activity, and (2) both GTP- and GDP-locked Arl8b mutants display similar levels of accumulation on *Npc1*^{-/-} lysosome membranes (**Figures 5E and 5F**), suggesting that Arl8 may be sequestered in an inactive GDP-bound form. Because SKIP binding to Arl8 is nucleotide dependent (Rosa-Ferreira and Munro, 2011), we further tested possible disruption of Arl8-SKIP interaction in *Npc1*^{-/-} and found that an anti-Arl8 antibody co-precipitated less SKIP in *Npc1*^{-/-} brain homogenates compared with WT ($p = 0.0049$; **Figures 5G and 5H**). Collectively, these results suggest that the aberrant sequestration of kinesin-1 and Arl8 on *Npc1*^{-/-} lysosomal membranes impairs the assembly of the motor-adaptor-effector complexes that drive lysosome delivery into axons.

Elevated Lysosomal Membrane Cholesterol Sequesters Kinesin-1 and Arl8

A recent study established that the limiting membrane of *Npc1*^{-/-} lysosomes in non-neuronal cells shows significant cholesterol accumulation (Lim et al., 2019). This prompted us to examine whether changes in lipid composition in the lysosomal membrane contribute to the aberrant sequestration of kinesin-1 and Arl8. We first determined whether *Npc1*^{-/-} neurons show elevated lysosomal membrane cholesterol by applying the cholesterol biosensor GST-D4H*-mCherry to semi-permeabilized cells (Lim et al., 2019; Wilhelm et al., 2017), which allows the D4H* protein to selectively bind cholesterol-rich intracellular membranes with a cholesterol molar content that exceeds 10% (Maekawa and Fairn, 2015). In WT neurons, D4H*-mCherry appeared mostly diffuse and did not bind LAMP1-labeled endolysosomal membranes (**Figure 6A**), indicating that their membrane cholesterol content is below 10%. However, *Npc1*^{-/-} neurons displayed strong vesicular D4H*-mCherry signals overlapping LAMP1-labeled endolysosomes, reflecting elevated cholesterol content within *Npc1*^{-/-} lysosomal membranes compared with WT ($p = 0.0008$; **Figure 6B**). We also co-labeled free cholesterol with filipin and observed increased luminal cholesterol levels in *Npc1*^{-/-} relative to WT ($p < 0.0001$; **Figures 6A and 6C**), indicating that *Npc1*^{-/-} neuronal

lysosomes accumulate both luminal and membrane cholesterol. Notably, the vast majority of vesicular D4H*-mCherry signals in *Npc1*^{-/-} neurons overlapped with LAMP1, while its colocalization with SV2-labeled synaptic vesicles was hardly detectable and no significant change in their axonal distribution was observed (**Figures S4E-S4H**), suggesting that elevated membrane cholesterol may selectively impair lysosome transport in *Npc1*^{-/-} neurons.

We next sought to reduce lysosomal membrane cholesterol levels. Cyclodextrin derivatives have commonly been used to extract cholesterol from membranes (Kilsdonk et al., 1995; Ohvo and Slotte, 1996; Zidovetzki and Levitan, 2007). Interestingly, the cyclodextrin derivative HPCD was shown to reduce luminal cholesterol, delay disease onset, and extend lifespan in *Npc1*^{-/-} mice (Davidson et al., 2009; Liu et al., 2008; Liu et al., 2009), supporting its rationale for evaluation in a clinical trial with NPC patients (Ory et al., 2017). Treatment with low doses of 100- and 1000- μ M HPCD for 24 h mobilized luminal cholesterol from endolysosomes without toxicity to *Npc1*^{-/-} mouse neurons (Peake and Vance, 2012). However, it remains to be determined whether HPCD can effectively remove membrane cholesterol from *Npc1*^{-/-} lysosomes. Strikingly, treatment of *Npc1*^{-/-} cortical neurons with HPCD (100 μ M for 48 h) nearly abolished D4H*-mCherry signals on lysosome membranes ($p < 0.0001$) and also significantly reduced luminal cholesterol ($p < 0.0001$) in lysosomes (**Figures 6D-6F**). STED imaging further revealed that treatment with HPCD released Arl8 and kinesin-1 sequestration from the surface of *Npc1*^{-/-} lysosomes, returning to a cytosolic distribution similar to that observed in WT neurons (**Figures 6G and 6H**).

As a complementary approach, we magnetically isolated endolysosomes to further examine the altered distribution of the kinesin-1-SKIP-Arl8 complex on *Npc1*^{-/-} lysosomes. Neuronal lysosomes were loaded with iron-dextran and separated by magnetic chromatography (Bilgin et al., 2017; Diettrich et al., 1998), followed by immunoblotting to assess relative protein levels within endolysosomal membranes. LAMP1, but not GAPDH, TOM20, or Sec61 β , was readily detected in captured organelles, indicating an enriched endolysosome population following magnetic chromatography. Consistent with results observed by STED imaging, lysosomal membranes showed increased levels of Arl8 and kinesin-1, but not SKIP, in *Npc1*^{-/-} neurons compared with WT, further supporting their non-functional accumulation in the absence of the GTPase effector SKIP. Importantly, HPCD treatment reduced the abnormal accumulation of Arl8 and kinesin-1 on endolysosome membranes of *Npc1*^{-/-} neurons (**Figures 6I and 6J**), further suggesting cholesterol-mediated sequestration. These changes occurred without noticeable differences in the total protein levels in whole cell lysates, suggesting that the observed alterations occur locally

at *Npc1*^{-/-} lysosomal membranes. Collectively, these STED imaging and lysosome purification analyses consistently suggest that altered lipid composition in *Npc1*^{-/-} lysosomal limiting membranes drives the aberrant sequestration of kinesin-1 and Arl8.

Reduction of Lysosomal Membrane Cholesterol Rescues Axonal Lysosome Delivery and Reduces Autophagic Stress in *Npc1*^{-/-} Neurons

We next determined whether releasing kinesin-1/Arl8 sequestration by reducing lysosomal membrane cholesterol has an impact on axonal lysosome density in DRG neurons isolated from presymptomatic (P30-40) *Npc1*^{-/-} mice. Neurons were incubated with HPCD for 48 h, followed by co-immunostaining at DIV3-4. In WT neurons, HPCD treatment did not alter axonal lysosome density compared with H₂O control ($p > 0.9999$; **Figures 7A and 7B**). However, *Npc1*^{-/-} neurons treated with HPCD showed a striking increase in the number of axonal lysosomes (*Npc1*^{-/-} H₂O: 8.54 ± 1.02 ; *Npc1*^{-/-} HPCD: 15.20 ± 1.44 ; $p = 0.0014$; per 100- μ m axon length; **Figures 7A and 7B**), suggesting that reducing lysosomal membrane cholesterol effectively rescues lysosome transport into axons from presymptomatic *Npc1*^{-/-} mice.

We directly tested this possibility by examining the anterograde transport of lysosomes from the soma to distal axons in *Npc1*^{-/-} neurons following HPCD treatment. Neurons were cultured in microfluidic devices, and only the soma/dendritic chamber was briefly loaded with MDW933 for 15 min. Neurons were then washed and fixed after 0, 1, 2, or 3 h, followed by imaging growth cones within the axon chamber. Lysosomes visualized in distal axon terminals following this soma-restricted labeling represent those transported there from the cell body. Remarkably, as post-wash time increased from 0 to 3 h, HPCD treatment resulted in a more rapid accumulation of active GCase signal at growth cones compared with H₂O-treated *Npc1*^{-/-} neurons (**Figures 7C and 7D, Videos S7, S8**), reflecting enhanced axonal delivery of lysosomes from the cell body.

Lysosome transport to distal axons is critical for maintaining efficient autophagic flux (Farfel-Becker et al., 2019). Given that lysosome transport defects precede axonal AV accumulation in *Npc1*^{-/-} neurons (**Figures 3B-3J, S3E and S3F**), we next determined whether rescuing axonal lysosome delivery with HPCD treatment has an impact on autophagic stress in *Npc1*^{-/-} axons. To test this, DRG neurons from P30-40 *Npc1*^{-/-} mice were nucleofected with EGFP-LC3 at DIV0, treated with HPCD for 48-72 h, then incubated with MDW941 prior to live imaging at DIV3-4. Treatment of *Npc1*^{-/-} neurons with HPCD resulted in increased axonal lysosome density (H₂O: 5.22 ± 0.60 ; HPCD: 12.47 ± 1.24 ; $p < 0.0001$; per 100- μ m axon length) and reduced axonal AV

density (H_2O : 8.32 ± 0.62 ; HPCD: 3.28 ± 0.45 ; $p < 0.0001$; per 100- μm axon length) (**Figures 7E and 7F**), suggesting that reducing lysosomal membrane cholesterol and therefore enhancing axonal lysosome delivery facilitates autophagic flux in presymptomatic *Npc1*^{-/-} DRG axons.

Elevated Arl8b Expression Facilitates Lysosome Transport into Axons and Reduces Autophagic Stress and Neuronal Death in Presymptomatic *Npc1*^{-/-} Neurons

Kinesin-1 requires SKIP to drive lysosome transport into axons (Farias et al., 2017), and SKIP requires Arl8 in its active GTP-bound state for binding (Rosa-Ferreira and Munro, 2011). Because SKIP did not show enhanced lysosomal membrane association and displayed reduced interaction with Arl8 in *Npc1*^{-/-} mouse brains (**Figures 5G and 5H**), Arl8 may be sequestered in an inactive GDP-bound form. We tested whether elevated Arl8b expression in *Npc1*^{-/-} neurons is able to overcome the cholesterol-linked sequestration of endogenous Arl8 and recruit SKIP to rescue lysosome transport into axons. DRG neurons isolated from presymptomatic (P30-40) *Npc1*^{-/-} mice were nucleofected with Arl8b-mCh at DIV0, followed by co-immunostaining at DIV3-4. Strikingly, elevated Arl8b expression rescued lysosome density in *Npc1*^{-/-} axons (*Npc1*^{-/-}: 8.58 ± 0.91 ; *Npc1*^{-/-} with Arl8b-mCh: 29.73 ± 3.18 ; $p < 0.0001$; per 100- μm axon length) and also further increased the number of lysosomes in WT axons (WT: 18.80 ± 1.50 ; WT with Arl8b-mCh: 32.11 ± 2.56 ; $p = 0.0156$; per 100- μm axon length) (**Figures 7G and 7H**). The effect of Arl8b-mCh on axonal lysosome density was nucleotide dependent, as expressing the GTP-locked, but not GDP-locked, form of Arl8b-mCh increased lysosome density in both WT and *Npc1*^{-/-} axons (**Figures S5A and S5B**). Concordantly, elevated Arl8b-mCh expression in *Npc1*^{-/-} neurons reduced lysosomal accumulation in the soma (**Figures S5E and S5F**). Notably, high levels of Arl8b overexpression resulted in axonal organelle accumulation (**Figure S6**), highlighting that maintaining a steady-state distribution of axonal lysosomes is critical for axonal homeostasis (Gowrishankar et al., 2017).

We next asked whether facilitating lysosome transport to distal axons by elevating Arl8b expression also reduces AV accumulation in *Npc1*^{-/-} axons. DRG neurons cultured from presymptomatic *Npc1*^{-/-} mice were nucleofected with EGFP-LC3 alone or together with Arl8b-mCh at DIV0, followed by live imaging at DIV3-4. Consistently, elevated Arl8b expression reduced AV density in *Npc1*^{-/-} axons (*Npc1*^{-/-}: 9.29 ± 0.57 ; *Npc1*^{-/-} with Arl8b-mCh: 4.93 ± 0.35 ; $p < 0.0001$; per 100- μm axon length) (**Figures 7I and 7J**). Importantly, this reduction was also dependent on Arl8 GTPase activity (**Figures S5C and S5D**). Together, these experiments suggest that facilitating

lysosome transport into *Npc1*^{-/-} axons is sufficient to support the maturation and clearance of axonal AVs at this early presymptomatic stage.

We further examined whether elevated *Arl8b* expression has an impact on the survival of *Npc1*^{-/-} cortical neurons isolated from presymptomatic adult mice (P30-40) by performing terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays. *Npc1*^{-/-} neurons showed increased TUNEL-positive neuron death compared with WT at DIV7 (WT: $18.81 \pm 0.64\%$; *Npc1*^{-/-}: $26.96 \pm 0.80\%$; $p < 0.0001$) and a further increase at DIV10 (WT: $22.55 \pm 0.45\%$; *Npc1*^{-/-}: $35.62 \pm 0.67\%$; $p < 0.0001$), indicating progressive apoptosis with age in culture. However, elevated *Arl8b* expression reduced *Npc1*^{-/-} neuron death at DIV7 and DIV10, restoring neuron survival to levels observed in WT neurons (**Figures 7K and 7L**). Taken together, these results support a model in which a cholesterol-linked impairment of lysosome transport to distal axons disrupts autophagic maturation and clearance, thus contributing to axon degeneration in *Npc1*^{-/-} neurons.

DISCUSSION

In this study, we reveal a lipid-mediated mechanism contributing to axonal dystrophy in NPC neurons. Abnormal cholesterol accumulation within NPC lysosome membranes in the soma impairs their axonal delivery and positioning by sequestering kinesin-1 and Arl8 independent of SKIP, thus disrupting axonal degradation capacity contributing to dystrophy and degeneration. The biological insights revealed in this study advance our understanding of early axonal dystrophy in NPC and shed light on the recent translational application of HPCD in NPC patients (Ory et al., 2017).

Coordinated Bidirectional Transport of Lysosomes Maintains Axonal Homeostasis

Axonal dystrophy and autophagic stress are two major pathological hallmarks of common neurodegenerative diseases and LSDs associated with lysosome dysfunction (Beard et al., 2017; Bordi et al., 2016; Gowrishankar et al., 2015; Haidar and Timmerman, 2017; Lee et al., 2011; Micsenyi et al., 2009; Tagliaferro and Burke, 2016; Walkley et al., 2010; Xie et al., 2015; Yang et al., 2013; Zigdon et al., 2017). These studies raised a fundamental question as to whether reduced clearance of endo-lysosomal-autophagic organelles contributes to axon pathology and dystrophy. Since degradative lysosomes have been thought to be confined to the neuronal cell body, it was unclear why a disturbance in the lysosomal system would be associated with axonal pathology in neurodegenerative LSDs such as NPC (Walkley et al., 2010). In agreement with previous reports (Boland and Platt, 2015; Walkley et al., 2010; Walkley and Suzuki, 2004), dystrophic axons in NPC did not contain the characteristic lysosome inclusions representative of NPC lysosomes. Rather, we observed a predominant accumulation of autophagic vacuoles, multivesicular bodies, and multilamellar vesicles, largely representing intermediates of endocytic and autophagic pathways. It has been well established that the retrograde transport of axonal AVs is linked to their maturation and degradation (Lee et al., 2011; Maday et al., 2012). One of our most unexpected observations was that despite increased axonal autophagic stress, AVs in NPC axons displayed similar motility patterns compared with control axons. Given global disruptions to axonal transport would lead to accumulation of a more diverse set of organelles, these findings suggested that impaired maturation and clearance of lysosomal substrates likely underlies early axonal pathology in NPC.

Our recent study in microfluidic devices demonstrated that soma-labeled degradative lysosomes are dynamically delivered to distal axons, where they target to AVs, facilitating their maturation

into autolysosomes by supplying active lysosomal hydrolases (Farfel-Becker et al., 2019). We propose that both the anterograde delivery of lysosomes from the soma to distal axons, as well as the retrograde trafficking of AVs generated at axon terminals, are essential to achieve effective autophagic flux in axons. Consistent with this concept, we observed that NPC neurons display a reduced number of lysosomes delivered into distal axons, thus impairing maturation and turnover of AVs during their retrograde transport route. This concept is supported by several studies showing that impairments in either transport process lead to axonal autophagic stress and neurodegeneration (Cheng et al., 2015; Farfel-Becker et al., 2019; Farias et al., 2017; Tammineni et al., 2017; Wong and Holzbaur, 2014). Together, our findings conceptually advance current knowledge and highlight that efficient autophagic clearance in axons requires coordination between their retrograde transport and the anterograde delivery of soma-derived degradative lysosomes to distal axons. This bidirectional transport model facilitates efficient degradation and removal of axonal cargos during their opposing trafficking routes.

Altered Membrane Lipid Composition Impairs Lysosome Delivery to NPC Axons

Cholesterol accumulation in the lumen of NPC lysosomes was reported to alter lysosome positioning through the Rab7/RILP/ORP1L (Rocha et al., 2009) or TMEM55B/JIP4 pathway (Willett et al., 2017) as well as impair Rab7 and kinesin motor function (Lebrand et al., 2002), highlighting the pathological role of cholesterol accumulation in altering endolysosomal transport. However, the mechanisms and pathological relevance in NPC neurons and its contribution to axonal dystrophy and autophagic stress remained largely unknown. Here we observed that axonal lysosomes in NPC display a selective defect in anterograde transport without affecting their retrograde motility, resulting in a net reduction in the lysosome density in NPC axons that impeded efficient axonal cargo clearance.

Defective axonal lysosome delivery suggested a disruption to the kinesin-1-SKIP-Arl8 transport complex in NPC. To elucidate mechanisms underlying impaired lysosome transport into axons, we investigated recruitment of this complex to lysosomes using super-resolution imaging and biochemical endolysosomal isolations. Consistent with our notion that elevated lipid content within lysosomal membranes alters anterograde lysosome transport, we observed a disordered accumulation of kinesin-1 and Arl8 on NPC lysosome membranes, which occurs independent of the kinesin cargo-binding domain, the motor-adaptor effector SKIP, and the nucleotide status of Arl8b. Such lipid-mediated Arl8 sequestration disrupted formation of the motor-adaptor-effector complex, thus impairing lysosome transport to NPC distal axons. Notably, the Ragulator complex

negatively regulates the BORC complex in non-neuronal cells (Filipek et al., 2017; Pu et al., 2017) and functions upstream of the kinesin-1-SKIP-Arl8 complex (Pu et al., 2015). Disrupting Ragulator by Lamtor1 knockdown in P30-40 WT DRG neurons did not result in an increase in axonal lysosomes (data not shown), suggesting that other neuron-specific complexes may regulate the BORC-dependent axonal trafficking of endolysosomes. However, the lipid-mediated disruption of the kinesin-1-SKIP-Arl8 transport complex in NPC was further supported by application of HPCD, which reduced membrane cholesterol and released Arl8 and kinesin-1 sequestration, thereby rescuing axonal lysosome delivery and reducing autophagic stress in presymptomatic NPC axons.

Cyclodextrin was reported to reduce lysosomal sphingolipid accumulation (Hoque et al., 2020) and reduce cholesterol storage through lysosomal exocytosis (Chen et al., 2010). It is possible that elevated membrane sphingolipid content may also contribute to the lipid-mediated motor-adaptor sequestration on NPC lysosomes. Levels of cholesterol and sphingolipids in membranes influence their properties and organization into liquid-ordered and -disordered lipid phases (Elson et al., 2010). Recent structural analyses of the yeast orthologue of NPC1 revealed a hydrophobic tunnel within it that moves cholesterol and other sterols from the lysosomal lumen to the inner leaflet of the limiting membrane (Winkler et al., 2019). While it is well established that cholesterol stores in the lumen of NPC lysosomes, it was recently reported that NPC lysosomes also accumulate ER-derived cholesterol at the limiting membrane (Lim et al., 2019), which may be distinguished from cholesterol from an endocytic source.

At physiological levels, cholesterol-enriched membrane domains play an important role in supporting various signaling and membrane trafficking events (Epand, 2006; Mukherjee and Maxfield, 2004). Proteins may preferentially associate and sequester into cholesterol-enriched regions of the membrane for their normal function (Ge et al., 2018; Rahbek-Clemmensen et al., 2017). However, abnormally elevated cholesterol levels were reported to alter SNARE complex assembly (Fraldi et al., 2010) and interfere with the GDP-GTP cycle of small GTPases (Ganley and Pfeffer, 2006; Lebrand et al., 2002), indicating a pathological role for excess membrane cholesterol in driving aberrant protein sequestration. Because cholesterol is an important regulator of membrane organization, cells must maintain cholesterol levels within a narrow range. Therefore, treatment with cyclodextrin derivatives may require careful dosing, as excessive depletion of cholesterol from cellular membranes may alter cholesterol-dependent homeostatic events (Peake and Vance, 2012; Sarkar et al., 2013; Yang et al., 2017).

Lysosomal Dysfunction and Axonal Autophagic Stress in NPC Neurons

Reducing lysosomal degradation capacity by leupeptin or bafilomycin A1 treatment results in AV accumulation in axons, neurites, and/or the soma (Boland et al., 2008; Lee et al., 2011; Maday and Holzbaur, 2016). These studies suggest that reduced lysosomal proteolytic function observed in NPC cells (Elrick et al., 2012) may also contribute to autophagic stress in NPC axons. Impaired AV-lysosome fusion was also shown to induce AV accumulation in NPC1-deficient cells (Maetzel et al., 2014; Sarkar et al., 2013). Studies in *Npc1*^{-/-} mouse brains demonstrated AV accumulation (Ko et al., 2005) revealed to be mTOR-independent (Boland et al., 2010), suggesting that autophagic stress may not result from increased autophagy induction in the brain. Interestingly, a recent study in an NPC cat model reported accumulation of LC3-positive puncta in axons and presynaptic terminals but not in the neuronal cell body or dendrites (Gurda et al., 2018), further highlighting the importance of the axonal trafficking and positioning of mature lysosomes to maintain autophagic flux and local degradation capacity. These studies support our bidirectional transport model in which axonal AVs achieve effective maturation and clearance during their retrograde trafficking route along axons, where they accept active lysosomal hydrolases through fusion with mature lysosomes moving anterogradely from soma-derived pools.

While progressive lipid storage in NPC alters lysosome degradation capacity with disease progression, we unexpectedly observed a reduced density of degradative lysosomes in axons but an expanded lysosomal system in the cell body in embryonic NPC cortical neurons at DIV7-8, which preceded axonal AV accumulation. Subsequently, in DRG neurons isolated from adult presymptomatic NPC mice, decreased axonal lysosome density was associated with a further reduction in active lysosomes and a robust increase in axonal AVs. Consistent with these observations, rescuing lysosome delivery to distal axons with elevated *Arl8b* expression reduced, but did not eliminate, autophagic stress in presymptomatic NPC axons. Therefore, proper lysosome positioning and degradation capacity are both important to maintain axonal homeostasis.

Limitations of the Study

While our study supports a cholesterol-dependent sequestration of kinesin-1 and Arl8 on the surface of NPC lysosomes, we do not rule out that changes in other membrane lipids may also contribute to this abnormal protein sequestration. Further development of tools for visualizing diverse lipid species in live cells at high spatiotemporal resolutions may help determine the lipid environment leading to protein sequestration on NPC lysosomes and further advance our understanding of the dynamic interplay between lipids and proteins in mediating cellular events at the lysosomal membrane.

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Author Contributions: J.C.R. designed and performed cell biology experiments and analyzed data; S.L. performed STED imaging; T.F-B. performed lysosome delivery assays; N.H. generated kinesin and Arl8 mutants and performed biochemical assays; T.S. performed TUNEL assays; Y.X. performed EM imaging; X-T.C. performed the AV maturation assay and made the graphical abstract; M-Y.L. performed immunoblots; F.M.P. proposed experiments, interpreted data, and supervised the project; Z-H.S. is the senior author who conceived and directed the project; J.C.R. and Z-H.S. wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

Figure Legends

Figure 1. Aberrant AV Accumulation in Axons of *Npc1*^{-/-} Mice

(A, B) TEM images showing AV accumulation in DRG (A) and cortical (B) axons in *Npc1*^{-/-} mice at symptomatic stages (P50-60). Such accumulations were not readily observed in age-matched WT mice. Colored boxes represent four-fold higher magnification of corresponding regions in upper panels (green: WT; orange: *Npc1*^{-/-}).

(C, D) Images (C) and analysis (D) showing a robust increase in AV density in DRG axons isolated from presymptomatic *Npc1*^{-/-} mice (P30-40) compared with WT neurons at the same age.

(E, F) Kymographs (E) and analysis (F) showing no significant difference in the motility of axonal AVs between WT and *Npc1*^{-/-} neurons. See also **Videos S1 and S2**. DRG neurons isolated from presymptomatic *Npc1*^{-/-} mice (P30-40) were nucleofected with EGFP-LC3 at DIV0, followed by live imaging at DIV3-4.

Data were collected from the total number of axons indicated above bars (D) or vesicles (v) from the number of axons (n) indicated in parentheses (F), presented as mean ± SEM with dots representing individual axons, and analyzed by Student's *t* test. Scale bars: 800 nm (A, B), 10 μm (C, E). See also **Figure S1**.

Figure 2. Reduced Axonal Density of Lysosomes in *Npc1*^{-/-} Neurons

(A-D) Images (A, C) and analyses (B, D) showing reduced density of active GCase-labeled degradative lysosomes and LAMP1-labeled endolysosomes in adult DRG neuron axons isolated from presymptomatic *Npc1*^{-/-} mice (P30-40). DRG neurons were incubated with MDW933 (500 nM) for 1 h to label active GCase, followed by immunostaining for β3-tubulin (A, B), or co-immunostained for LAMP1 and β3-tubulin (C, D).

(E-H) Images (E, G) and analyses (F, H) showing reduced density of degradative lysosomes and endolysosomes in axons of *Npc1*^{-/-} cortical neurons plated in microfluidic devices. At DIV8, neurons were loaded with MDW933 (500 nM) for 1 h to label active GCase, followed by β3-tubulin staining (E, F), or co-immunostained for LAMP1 and β3-tubulin (G, H).

(I-L) Images (I, K) and analyses (J, L) showing lysosomal accumulation in the soma of *Npc1*^{-/-} cortical neurons. Neurons at DIV8 were incubated with MDW933 to label degradative lysosomes followed by MAP2 immunostaining, or co-immunostained for MAP2 and LAMP1 to label endolysosomes (I, J), or incubated with BODIPY-FL-pepstatin A to label active cathepsin D prior to live imaging (K, L). Integrated density was measured in thresholded images using ImageJ, and data are presented as mean integrated density normalized to WT neurons.

(M, N) Immunoblots **(M)** and quantitative analysis **(N)** of LAMP1, GCase, and cathepsins B and D (CTSB and CTSD) in WT and *Npc1*^{-/-} cortical neurons at DIV7-8. Equal amounts of neuronal lysates (10 µg) were sequentially immunoblotted. Protein intensities of LAMP1, GCase, and both mature and immature forms of CTSB and CTSD were averaged from three repeats and normalized to WT.

Data were collected from the total number of axons **(B, D, F, H)** or neurons **(J, L)** indicated below or within bars, presented as mean ± SEM with dots representing individual values, and analyzed by Mann-Whitney test **(B)** or Student's *t* test **(D, F, H, J, L, N)**. Scale bars: 10 µm. See also **Figure S2**.

Figure 3. Impaired Axonal Delivery of Degradative Lysosomes in *Npc1*^{-/-} Neurons

(A) Schematic diagram of soma-restricted labeling of degradative lysosomes and their delivery to distal axons. Neurons are plated in the soma/dendritic chamber (1) of a microfluidic device that provides physical and fluidic separation of axons from cell bodies and dendrites. Only axons that enter the 450-µm-long microgrooves (2) are able to grow into the axon chamber (3). The soma/dendritic chamber is loaded with an activity-based lysosome probe for 30 min, followed by washes with imaging buffer and live imaging carried out in the axon chamber for 90 min.

(B-E) Images **(B, D)** and analyses **(C, E)** showing impaired delivery of active GCase- and cathepsin D (CTSD)-labeled degradative lysosomes from the soma chamber to distal axons in *Npc1*^{-/-} cortical neurons. At DIV7 and DIV10, soma-restricted labeling of degradative lysosomes was carried out by loading MDW941 (100 nM) **(B)** or BODIPY-FL-pepstatin A (1 µM) **(D)** for 30 min, followed by washes and live imaging in the axon chamber for 90 min. Data were quantified from the number of axons indicated in bars and normalized to WT.

(F-J) Kymographs **(F, H)** and analyses **(G, I, J)** showing impaired anterograde motility of degradative lysosomes in distal axonal segments of *Npc1*^{-/-} cortical neurons. Neurons plated in microfluidic devices were loaded with MDW941 (100 nM) **(F, G)** or BODIPY-FL-pepstatin A (1 µM) **(H, I)** for 30 min prior to live imaging at DIV8. Time-lapse images were collected every 2 s for 90 frames totaling 3 min. The percentage of anterograde (antero), retrograde (retro), or stationary (stat) lysosomes was quantified from the total number of vesicles (v) in the total number of axons (n) as follows: **(G)** WT: n=28 v=344, *Npc1*^{-/-}: n=29 v=226; **(I)** WT: n=36 v=433, *Npc1*^{-/-}: n=35 v=271. Active lysosome motility parameters including flux rate, speed, and the velocity in both directions were analyzed in axons of WT and *Npc1*^{-/-} cortical neurons **(J)**. See also **Videos S3 and S4**.

Data were analyzed by Student's *t* test (**C, E, I, J**) or Mann-Whitney test (**G**) and presented as mean \pm SEM alone (**C, E**) or with dots representing individual values (**G, I, J**). Scale bars: 10 μ m. See also **Figure S3, Videos S5 and S6**.

Figure 4. Aberrant Sequestration of Kinesin-1 and Arl8 on Lysosome Membranes in *Npc1*^{-/-} Neurons

(**A-F**) STED super-resolution images (**A, C, E**) and analyses (**B, D, F**) showing aberrant sequestration of endogenous kinesin-1 and its adaptor Arl8 on the surface of *Npc1*^{-/-} lysosome membranes in the soma. Enlarged views of the corresponding boxed regions are shown in (**A'**), (**C'**), and (**E'**). The percentage of LAMP1-labeled areas associated with Arl8 (**B**), kinesin-1 (**D**), or SKIP (**F**) were quantified and normalized to WT. Note that Arl8 and kinesin-1, but not SKIP, display enhanced distribution on the surface of *Npc1*^{-/-} lysosomes.

(**G-J**) STED images (**G, I**) and analyses (**H, J**) showing non-specific recruitment of truncated KHC-MD (motor domain, **G**) and KHC-CBD (cargo-binding domain, **I**) to *Npc1*^{-/-} lysosome membranes. WT and *Npc1*^{-/-} cortical neurons were transduced with GFP-tagged KHC-MD or KHC-CBD at DIV4, followed by co-immunostaining for GFP and LAMP1 at DIV8. Enlarged views of the corresponding boxed regions are shown in (**G'**) and (**I'**). The percentage of LAMP1-labeled areas associated with KHC-MD (**H**) or KHC-CBD (**J**) signals were quantified and normalized to WT.

Data were collected from the total number of neurons indicated within bars (**B, D, F, H, J**), presented as mean \pm SEM with dots representing individual neurons, and analyzed by Mann-Whitney test. Scale bars: 5 μ m.

Figure 5. Aberrant Accumulation of Kinesin-1 and Arl8 on *Npc1*^{-/-} Lysosome Membranes Is Independent of Arl8 GTPase Activity

(**A-D**) STED images (**A, C**) and analyses (**B, D**) showing kinesin-1 sequestration on *Npc1*^{-/-} lysosome membranes independent of Arl8 and SKIP. *Npc1*^{-/-} cortical neurons at DIV4 were transfected with control scrambled (scr), Arl8-siRNA (**A, B**), or SKIP-siRNA (**C, D**), followed by co-immunostaining for LAMP1 and kinesin-1 at DIV8. Pseudocolors were applied: magenta for LAMP1; cyan for kinesin-1. Enlarged views of the boxed regions are shown in (**A'**) and (**C'**). The percentage of LAMP1-labeled areas associated with kinesin-1 signals in *Npc1*^{-/-} cortical neurons transfected with Arl8-siRNA or SKIP-siRNA were quantified and normalized to *Npc1*^{-/-} neurons transfected with scr-siRNA. See also **Figures S4A-S4D**.

(**E, F**) STED images (**E**) and analyses (**F**) showing that kinesin-1 and Arl8 sequestration is not affected by Arl8 GTPase activity in *Npc1*^{-/-} neurons. *Npc1*^{-/-} cortical neurons were transfected with

WT, GTP-locked, or GDP-locked mutant forms of Arl8b-mCh at DIV4, followed by immunostaining for LAMP1, mCherry, and kinesin-1 at DIV8. Pseudocolors were applied. Enlarged views of the boxed regions are shown in (E'). The percentage of LAMP1-labeled areas associated with kinesin-1 or mCherry-tagged Arl8b signals in *Npc1*^{-/-} cortical neurons expressing Arl8b mutants were quantified and normalized to *Npc1*^{-/-} neurons expressing WT Arl8b.

(G, H) Co-immunoprecipitation **(G)** and analysis **(H)** showing reduced interaction of Arl8 and SKIP in *Npc1*^{-/-} mouse brain homogenates from E18 embryos. Protein band intensities were quantified using ImageJ and normalized to WT. Data were averaged from three repeats and analyzed by Student's *t* test.

Data were collected from the total number of neurons indicated within bars (**B, D, F**), presented as mean ± SEM with dots representing individual neurons, and analyzed by Mann-Whitney test (**B, D**) or one-way ANOVA, where *p* values represent comparisons of each condition against WT Arl8b (**F**). Scale bars: 5 μm.

Figure 6. Reducing Cholesterol Releases Sequestration of Kinesin-1 and Arl8 from *Npc1*^{-/-} Lysosome Membranes

(A-C) Images **(A)** and analyses **(B, C)** showing elevated membrane and luminal cholesterol in lysosomes of *Npc1*^{-/-} neurons at DIV7-8. Colocalization between D4H* and LAMP1 was measured and expressed as the fraction of LAMP1-labeled organelles that were also labeled by D4H* and normalized to *Npc1*^{-/-}. To quantify luminal cholesterol levels, the integrated density of filipin was measured in thresholded images and normalized to *Npc1*^{-/-}. See also **Figures S4E-S4H**.

(D-F) Images **(D)** and analyses **(E, F)** showing reduced cholesterol on endolysosome membranes and in the lysosomal lumen of *Npc1*^{-/-} neurons at DIV7-8 following HPCD treatment.

(G, H) STED images showing release of sequestered Arl8 **(G)** and kinesin-1 **(H)** from *Npc1*^{-/-} endolysosomal membranes by reducing lysosomal cholesterol with HPCD. Pseudocolors were applied: blue for GST-D4H*-mCherry; red for Arl8 **(G)** or kinesin-1 **(H)**. The edges of cell bodies and proximal processes are outlined with white dashed lines. Enlarged views of the boxed regions are shown in **(G')** and **(H')**.

(I, J) Sequential immunoblots **(I)** and quantitative analysis **(J)** showing cholesterol-dependent sequestration of kinesin-1 and Arl8 on *Npc1*^{-/-} neuronal lysosomes. Cortical neurons were treated with HPCD (100 μM) or H₂O control for 48 h, followed by magnetic isolation of lysosomes at DIV8. Equal amounts of whole cell lysates (5 μg) and captured endolysosomes (0.8 μg) were loaded and sequentially immunoblotted with antibodies as indicated. Protein band intensities were

quantified and averaged from three repeats, and data were normalized to H₂O-treated WT neurons.

Data were collected from the total number of neurons indicated below bars (**B, C, E, F**), presented as mean \pm SEM with dots representing individual images (**B, E**) or neurons (**C, F**), and analyzed by Mann-Whitney test (**B, C**), Student's *t* test (**E, F**), or one-way ANOVA (**J**). ** $p < 0.01$. Scale bars: 10 μ m (**A, D**), 2 μ m (**G, H**).

Figure 7. Cholesterol Reduction and Arl8b Expression Rescue Lysosome Transport into Axons of *Npc1*^{-/-} Neurons

(**A, B**) Images (**A**) and analysis (**B**) showing rescued axonal lysosome density in presymptomatic *Npc1*^{-/-} DRG neurons by reducing membrane cholesterol with HPCD treatment.

(**C, D**) Images (**C**) and analysis (**D**) showing enhanced delivery of soma-labeled degradative lysosomes to distal axons following HPCD treatment in *Npc1*^{-/-} cortical neurons cultured in microfluidic devices. At DIV8, MDW933 (500 nM) was loaded to the soma/dendritic chamber for 15 min. Neurons were then washed and fixed after 0, 1, 2, or 3 h, followed by immunostaining for β 3-tubulin. Axonal terminals are outlined with white dashed lines. See also **Videos S7 and S8**.

(**E, F**) Images (**E**) and analyses (**F**) showing reduced density of axonal AVs and increased density of lysosomes with HPCD treatment in DRG axons from P30-40 *Npc1*^{-/-} mice.

(**G, H**) Images (**G**) and analysis (**H**) showing rescued axonal lysosome density with elevated Arl8b expression in DRG axons from P30-40 *Npc1*^{-/-} mice.

(**I, J**) Images (**I**) and analysis (**J**) showing reduced axonal autophagic stress with elevated Arl8b expression in DRG neurons from presymptomatic *Npc1*^{-/-} mice at P30-40.

(**K, L**) Images (**K**) and analysis (**L**) showing ameliorated *Npc1*^{-/-} neuron death following Arl8b overexpression. Cortical neurons isolated from presymptomatic mice (P30-40) were transduced at DIV0 with Arl8b-mCh or mCh. TUNEL assays were performed at DIV7 and DIV10, and the percentage of TUNEL-positive cells to total DAPI staining was calculated in thresholded images (705 x 705 μ m) using ImageJ.

Data were collected from the total number of axons (**B, F, H, J**) or images (**L**) as indicated, or from > 30 axon terminals for each time point (**D**), presented as mean \pm SEM with dots representing individual axons, and analyzed by one-way ANOVA (**B, H, L**) or Student's *t* test (**D, F, J**). ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Scale bars: 10 μ m (**A, E, G, I**), 5 μ m (**C**), and 50 μ m (**K**). See also **Figures S5 and S6**.

STAR Methods

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Zu-Hang Sheng (shengz@ninds.nih.gov).

Materials Availability

Plasmids newly generated in this study are listed in the key resources table and available on request.

Data and Code Availability

The datasets supporting the current study are available from the corresponding author on request.

Experimental Model and Subject Details

Npc1 Mouse Model and Care

Npc1 null (BALB/cNctr-*Npc1*^{m1N/J}; *Npc1*^{-/-}) and WT (*Npc1*^{+/+}) mice were generated from heterozygote matings, and breeder pairs were kindly provided by Dr. William Pavan (Loftus et al., 1997). Animals were maintained in the NINDS Animal Facility, where they were housed in groups of 3-5 mice per cage on a 12-hour light-dark cycle (6 am – 6 pm) with access to food and water *ad libitum*. Primary neurons were obtained from mouse embryos at embryonic day 18-19 or adult mice at postnatal day 30-40, and animals of both sexes were used. All animal care and procedures were performed in accordance with NIH guidelines and approved by the NIH, NINDS/NIDCD Animal Care and Use Committee.

Primary Neuron Cultures

For embryonic cortical neurons, embryos from timed heterozygote breedings were obtained at embryonic day 18-19 and stored in ice-cold Hibernate medium supplemented with 2% B27 and 0.1X antibiotic-antimycotic while genotyping was performed to identify WT and *Npc1*^{-/-} embryos. Following identification, cortical neurons were prepared as previously described (Kang et al., 2008). Cortical neurons were cultured in Neurobasal media supplemented with 2% B27, 0.5 mM GlutaMAX, and 55 μ M 2-mercaptoethanol and maintained at 37°C and 5% CO₂. Media was one-half changed at DIV1 and one-fourth changed every three days after. For adult cortical neurons,

cultures were prepared from mice at postnatal day 30-40 (P30-40) using the Adult Brain Dissociation Kit according to the manufacturer's instructions.

For DRG neurons, WT and *Npc1*^{-/-} littermates at P30-40 were anesthetized with intraperitoneal injection of 2.5% avertin then transcardially perfused with ice-cold perfusion buffer (125 mM NaCl, 25 mM NaHCO₃, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 10 mM D-glucose, 2 mM MgCl₂, 2 mM CaCl₂ bubbled with 95% O₂ and 5% CO₂). After bisecting the spinal canal to expose the spinal cord, DRGs were removed and transferred to HBSS supplemented with 10 mM HEPES and 0.1X antibiotic-antimycotic. After excess dorsal roots were trimmed off, DRGs were digested in 2.5 units/mL dispase II and 200 units/mL collagenase at 37°C for 30 minutes followed by 35 minutes gently rotating at room temperature. DRGs were triturated in Neurobasal-A media supplemented with 2% B27, 0.5 mM GlutaMAX, and 2.5% fetal bovine serum. After filtering debris with a 70-µm nylon strainer, neurons were collected and plated on 12-mm coverslips coated with 20 µg/mL poly-L-ornithine and 10 µg/mL laminin. Where indicated and before plating, DRGs were nucleofected with 0.4 µg plasmid DNA using a Basic Neuron Small Cell Number Nucleofector Kit according to the manufacturer's instructions. DRG neurons were maintained at 37°C and 5% CO₂, and media was one-half changed three hours after plating.

Method Details

Lentivirus Production

Lenti-X 293T cells were transfected with the desired lentiviral construct, the packaging plasmid psPAX2 (Addgene #12260), the envelope plasmid pMD2.G (Addgene #12259), and pAdVantage for enhanced protein expression at a ratio of 11:7.5:2.5:1. DMEM media was changed every 24 hours and collected at 48- and 72-hours post-transfection. Following centrifugation to remove cell debris, lentivirus-containing media was aliquoted and stored at -80°C until use.

Microfluidic Device Preparation and Culture

Microfluidic devices were prepared as previously described (Zhou et al., 2016). Briefly, SYLGARD 184 silicone elastomer base was combined with its corresponding curing agent at a ratio of 10:1. After mixing and de-foaming, the silicone elastomer was poured into a silicone wafer template made out of SU-8 by photolithography. Following desiccation for 2.5 hours to remove air bubbles, the wafer was heated at 80°C for 2 hours to cure. Then, the silicone elastomer was carefully removed from the wafer, and microfluidic devices were punched out following template guides.

The devices were then washed in an ultrasonic cleaner consecutively for 10 minutes each in 50% ethanol followed by double-distilled water. Once dry, microfluidic devices were adhered to coverslips prior to neuron culture.

For culture in microfluidic devices, 2×10^5 freshly dissociated cortical neurons in a volume of 10 μl were added directly to the soma/dendritic transwell. If necessary, for lentiviral transduction, 5 μl of the desired lentivirus was combined with 2×10^5 freshly dissociated neurons in a volume of 5 μl , and this 10 μl volume was then added directly to the soma/dendritic transwell. To equilibrate, 10 μl of media was added directly to the axon transwell. Following a 10-minute incubation, 200 μl of media was added to the axon chamber followed by 180 μl of media to the soma/dendritic chamber. After a one-fourth media change at DIV1, media was one-fourth changed every three days.

Labeling Degradative Lysosomes with Activity-based Lysosome Probes

For labeling active glucocerebrosidase (GCase), green (MDW933) and red (MDW941) fluorescent activity-based probes specific for GCase (Farfel-Becker et al., 2019; Witte et al., 2010) were kindly provided by Dr. Ellen Sidransky (Westbroek et al., 2016). Unless otherwise stated, live neurons were incubated with 500 nM MDW933 or 100 nM MDW941 for 30 minutes or 1 hour at 37°C and 5% CO₂ followed by live-neuron imaging or fixation in Bouin's solution with 4% sucrose for immunostaining. For labeling active cathepsin D (CTSD), live neurons were incubated with 1 μM BODIPY-FL-pepstatin A for 30 minutes at 37°C and 5% CO₂ followed by live-neuron imaging.

Live-neuron Imaging and Motility Analysis

For live-cell imaging, neurons were rinsed three times and transferred to low fluorescence Hibernate medium supplemented with 2% B27 and 0.5 mM GlutaMAX. Cells were visualized with a 40x/1.3 NA oil immersion objective on a Zeiss LSM880 confocal microscope. Time-lapse imaging was performed in an incubation chamber maintained at 37°C. To quantify motility, kymographs were generated using ImageJ as previously described (Kang et al., 2008). Organelles were considered stationary if the net displacement was $\leq 10 \mu\text{m}$ during the entire acquisition period. If the net displacement was $\geq 10 \mu\text{m}$ throughout this period, the organelle was considered motile in its corresponding direction.

Immunofluorescence

Neurons were plated on poly-L-ornithine-coated 12-mm coverslips in 24-well plates at a density of 0.2×10^6 cells per well or in microfluidic devices as indicated above. At DIV7-8, neurons were quickly rinsed in PBS then fixed with either 4% paraformaldehyde (PFA) with 4% sucrose or Bouin's solution with 4% sucrose for 15 minutes at room temperature. Cells were then washed three times with PBS, permeabilized and blocked with 0.4% saponin, 1% BSA, and 5% goat serum in PBS for 1 hour, and incubated with primary antibodies in 0.1% saponin, 1% BSA, and 5% goat serum in PBS overnight at 4°C. Alternatively, after fixation, cells were washed three times with PBS, permeabilized with 0.1% Triton X-100 for 10 minutes, washed three times with PBS, blocked with 2% BSA and 5% goat serum in PBS for 1 hour, and incubated with primary antibodies in blocking buffer overnight at 4°C. After primary antibody labeling, cells were washed three times with PBS then incubated with Alexa Fluor conjugated secondary antibodies for 30 minutes at room temperature. After washing four times with PBS, coverslips were mounted with anti-fade mounting medium, while neurons in microfluidic devices were transferred to low fluorescence Hibernate medium prior to imaging.

Western Blot

Neurons were plated on poly-L-ornithine-coated 35-mm dishes at a density of 2×10^6 cells per dish. At DIV7-8, cells were rinsed with cold PBS and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% DOC) supplemented with a protease inhibitor cocktail tablet. Cell lysates were centrifuged at 15,000 x rpm for 15 minutes at 4°C and soluble proteins were collected. Equal amounts of proteins (10 µg) were resolved on NuPAGE 4-12% Bis-Tris protein gels and transferred to PVDF membranes under 250 mA constant current for 1.5 hours at 4°C. After blocking in 10% milk in TBS with 0.1% Tween 20 (TBST) for 1 hour at room temperature, membranes were incubated with primary antibodies overnight at 4°C. After washing four times with TBST, membranes were incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (1:5000) in 5% milk in TBST for 1 hour at room temperature. After washing four times with TBST, proteins were detected using SuperSignal chemiluminescent substrates and analyzed in ImageJ.

Electron Microscopy

Samples for EM were prepared as previously described (Xie et al., 2015) with minor modifications. Briefly, mice were anesthetized and transcardially perfused with freshly made EM fixative (2% glutaraldehyde and 2% paraformaldehyde in 0.1 N cacodylate buffer). Tissues were removed and post-fixed in EM fixative overnight, then sent to the NINDS EM Facility for staining and silver enhancement. After dehydration, embedding, and sectioning, images were taken using an electron microscope (1200EX; JEOL).

STED Super-resolution Imaging

STED super-resolution imaging was performed as previously described (Lin et al., 2017). Neurons were plated on 25-mm poly-L-ornithine-coated coverslips in 6-well plates and processed for GST-D4H-mCherry labeling and/or immunostaining. An inverted STED microscope with a resolution of ~50-90 nm (TCS SP8 STED 3X, Leica, Germany) was used. Alexa Fluor 488 was excited by a tunable white light laser (70% of maximum power) at 470 nm (10%) with the STED depletion laser at 592 nm (30% of the maximum power); its fluorescence at 480-560 nm was collected using time-gated detection (1.5-6.5 nsec). Alexa Fluor 594 was excited by the tunable white light laser at 594 nm (10%) with the STED depletion laser at 775 nm (30% of the maximum power); its fluorescence between 600-640 nm was collected using time-gated detection (1.5-6.5 nsec). GST-D4H*-mCherry was excited by the tunable white light laser at 568 nm (30%) with the STED depletion laser at 660 nm (30% of the maximum power); its fluorescence between 580-610 nm was collected using time-gated detection (1.5-6.5 nsec). Alexa Fluor 647 was excited by the tunable white light laser at 647 nm (20%) with the STED depletion laser at 775 nm (30% of the maximum power); its fluorescence between 660-750 nm was collected using time-gated detection (1.5-6.5 nsec). For dual-color and triple-color imaging, channels at the higher wavelength range were imaged first to avoid bleaching with a frame-scanning mode. Images were subjected to deconvolution processing using Huygens software.

Co-immunoprecipitation

To detect the interaction between SKIP and Arl8, an immunoprecipitation assay was performed. Brain tissues from WT and *Npc1*^{-/-} E18 embryos were collected and homogenized in lysis buffer (25 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 5% glycerol) supplemented with a protease inhibitor cocktail tablet and incubated on ice for 30 minutes. The crude homogenates were centrifuged at 13,000 x g at 4°C for 15 minutes. One milligram of cleared brain homogenates was incubated with anti-Arl8-coated Protein A Sepharose beads at 4°C for 3 hours, followed by

centrifugation and three washes with cold lysis buffer. Samples were eluted by heating at 95°C in sample buffer supplemented with 20 mM DTT, followed by western blotting analysis. The VeriBlot for IP detection reagent was used at 1:2000 as the secondary antibody to test IP signals without interference from the IP antibodies in the eluted samples.

Recombinant GST-D4H*-mCherry Protein Purification

Recombinant GST-D4H*-mCherry proteins were purified as previously described (Lim et al., 2019) with minor modifications. GST-D4H*-mCherry (Addgene #134604) was expressed in BL21(DE3) *Escherichia coli* and grown to an optical density at 600 nm (OD_{600}) of ~0.6. Protein production was then induced with IPTG at a final concentration of 0.4 mM and grown for 20 hours at 18°C. The bacterial pellet was resuspended in lysis buffer (20 mM Tris-Cl pH 8.0, 0.1 M NaCl, 1 mM DTT) supplemented with a protease inhibitor cocktail tablet and incubated with end-over-end rotation at 4°C for 30 minutes. The bacterial lysate was sonicated on an ice-cold water bath with a 5 second pulse at 30% amplitude followed by a 10 second break for a total of 15 minutes. Cleared lysate containing soluble protein was incubated with pre-equilibrated Glutathione Sepharose 4B beads for 2 hours at 4°C with end-over-end rotation. GST-D4H*-mCherry proteins were eluted with 25 mM reduced L-glutathione in 50 mM Tris-Cl pH 8.8 and 200 mM NaCl, then sequentially filtered using 3kDa and 30kDa molecular weight cutoff centrifugal filter units to concentrate the protein in 50 mM Tris-Cl pH 8.0 and 5 mM DTT. Following supplementation with sucrose to a final concentration of 0.5 M, the GST-D4H*-mCherry protein solution was aliquoted, frozen in liquid nitrogen, and stored at -80°C until use.

Detecting Luminal and Membrane Cholesterol with Filipin and GST-D4H*-mCherry

Luminal and membrane cholesterol was labeled as previously described (Lim et al., 2019; Wilhelm et al., 2017) with minor modifications. Neurons were plated on poly-L-ornithine-coated 12-mm coverslips in 24-well plates at a density of 0.2×10^6 cells per well. At DIV7-8, cells were quickly rinsed in PBS, then fixed with 4% PFA with 4% sucrose for 15 minutes at room temperature. The coverslips were washed three times with PBS, then submerged in liquid nitrogen for 30 seconds to permeabilize the plasma membrane while leaving intracellular membranes intact. Cells were blocked with 1% BSA in PBS for 1 hour, then incubated with recombinant GST-D4H*-mCherry (1:100) in 1% BSA in PBS for 2 hours at room temperature. The cells were quickly rinsed twice in PBS, re-fixed with 4% PFA with 4% sucrose for 10 minutes at room temperature, blocked with 1% BSA in PBS for 1 hour at room temperature, and incubated with a LAMP1 primary antibody overnight at 4°C. Cells were then washed three times with PBS,

and incubated with an Alexa Fluor 488 conjugated secondary antibody for 30 minutes at room temperature. After washing three times with PBS, cells were incubated with 50 $\mu\text{g/mL}$ filipin in PBS for 40 minutes at room temperature followed by three more washes in PBS. The coverslips were then mounted with anti-fade mounting medium.

Magnetic Isolation of Lysosomes

Lysosomes were isolated as previously described (Bilgin et al., 2017; Diettrich et al., 1998) with minor modifications. Neurons were plated on poly-L-ornithine- and laminin-coated 10-cm culture dishes at a density of 10×10^6 cells per dish. The day before isolation, conditioned neuronal media was collected by removing 10 mL media from each 10-cm dish. Cells were then treated with 1 mL DexoMAG iron-dextran solution (10 mg/mL) for 20 hours, washed with PBS, and cultured for an additional 2 hours in conditioned neuronal media collected from the day before. Cells were gently scraped and washed three times in cold PBS, then lysed using a 25 G needle in cold SCA buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl_2 , 1 mM EDTA, 1 mM EGTA, 250 mM sucrose) supplemented with 1 mM DTT, 1 mM Pefabloc, benzonase nuclease, and a protease inhibitor cocktail tablet. Ruptured cells were centrifuged at $800 \times g$ for 5 minutes at 4°C , then the supernatant containing cytosol and organelles was collected and this process repeated two more times, followed by loading onto pre-equilibrated MS columns fitted into the magnetic field of a MiniMACS separator attached to a MACS multistand. Columns were washed five times with SCA buffer, removed from the separator unit, and lysosomal membrane fractions were eluted with 200 μL buffer containing 5 mM Tris-HCl (pH 7.5), a protease inhibitor cocktail, and 1% Triton X-114. Protein concentration was determined using a BCA Protein Assay Kit according to the manufacturer's instructions. For immunoblots, eluted proteins were precipitated in methanol/chloroform, resuspended in LDS sample buffer with 100 mM DTT, and resolved on NuPAGE Bis-Tris protein gels.

RNA Extraction and Quantitative Real-Time PCR

Neurons were plated on poly-L-ornithine-coated wells of a 6-well plate at a density of 2×10^6 cells per well. At DIV7, total RNA was isolated using the RNeasy Mini Kit, and cDNA was synthesized using the iScript cDNA Synthesis Kit according to the manufacturer's instructions. Quantitative real-time PCR was performed using the iTaq Universal SYBR Green Supermix and a Bio-Rad CFX384 Real-Time system. Relative gene expression was normalized to β -actin. Oligonucleotide sequences were previously described (Chen et al., 2017; Mocholi et al., 2018) and are listed in the Key Resources Table.

TUNEL Assay and Analysis

Neurons were plated on 12-mm poly-L-ornithine-coated coverslips in 6-well plates. Where indicated and before plating, neurons were transduced with lentiviruses encoding Arl8b-mCh or mCh. At DIV7 and DIV10, cells were fixed and immunostained for MAP2 followed by TUNEL staining. TUNEL-positive neurons were detected using the Fluorescein *In Situ* Cell Death Detection Kit according to the manufacturer's instructions. The coverslips were then mounted with anti-fade mounting medium with DAPI. Neurons were visualized with a 40x/1.3 NA oil immersion objective on a Zeiss LSM880 confocal microscope. Images of entire coverslips were acquired using the Tiles acquisition mode setting in Zeiss ZEN black software. The percentage of TUNEL-positive cells to total DAPI staining was calculated in thresholded, watershed segmented images using ImageJ.

Image Acquisition and Quantification

Confocal images were acquired using a Zeiss LSM880 confocal microscope with a 40x/1.3 NA oil immersion objective. All images of endogenous proteins from a given experiment were taken on the same day using the same settings and analyzed using the same threshold. Image analyses were performed using ImageJ (Schindelin et al., 2012). For quantification of axonal autophagosome density, the number of EGFP-LC3 puncta was manually counted and normalized by axon length. For quantification of axonal lysosome density, the lysosome channel was segmented to identify bona fide lysosome vesicles. The fill holes and watershed functions were then applied, and the number of lysosomes was detected using the analyze particles function and normalized by axon length. For quantification of lysosomal integrated density in the cell body and axonal growth cones, the lysosome channel was segmented, and raw fluorescent intensity values were retrieved by redirecting the binary mask to the original 16-bit grayscale image. Mean integrated density values were calibrated to the area of the cell body or axonal growth cone and normalized to a reference group as indicated in the corresponding figure legend. For endolysosomal membrane cholesterol quantification, the Just Another Colocalization Plugin (JACoP) (Bolte and Cordelieres, 2006) was used. A set threshold was applied to each LAMP1 and GST-D4H*-mCh channel to identify authentic signals, and the Manders' overlap coefficient representing the fraction of LAMP1-vesicles that were also positive for GST-D4H*-mCh was calculated. Data were normalized to a reference group as indicated in the corresponding figure legend.

Quantification and Statistical Analysis

Statistical analyses were carried out using GraphPad Prism 8 software. For comparison between two groups, the Student's t test (sample size $n \geq 30$) or Mann-Whitney test (sample size $n < 30$) was used. For comparison between three or more groups, one-way ANOVA was used. All data are expressed as mean \pm SEM. Differences were considered statistically significant at a value of $p < 0.05$.

Supplementary Video Captions**Video S1. Axonal Transport of Autophagic Vacuoles in Distal Axons of WT DRG Neurons, Related to Figure 1E**

WT DRG neurons isolated from P30-40 mice were nucleofected with EGFP-LC3 at DIV0, followed by live imaging at DIV3-4. Time-lapse images were collected every 2 s for 150 frames totaling 5 min. Cell body is toward the left.

Video S2. Axonal Transport of Autophagic Vacuoles in Distal Axons of *Npc1*^{-/-} DRG Neurons, Related to Figure 1E

Npc1^{-/-} DRG neurons isolated from presymptomatic P30-40 mice were nucleofected with EGFP-LC3 at DIV0, followed by live imaging at DIV3-4. Time-lapse images were collected every 2 s for 150 frames totaling 5 min. Note the increased number of autophagic vacuoles in presymptomatic *Npc1*^{-/-} axons with similar motility patterns compared to control WT axons. Cell body is toward the left.

Video S3. Axonal Transport of Active Lysosomes in Distal Axons of WT Cortical Neurons, Related to Figure 3F

WT cortical neurons cultured in microfluidic devices were incubated with MDW941 (100 nM) for 30 min to label active GCase-positive lysosomes before live imaging at DIV7-8. Time-lapse images were collected every 2 s for 90 frames totaling 3 min. Distal axon terminal is toward the right.

Video S4. Axonal Transport of Active Lysosomes in Distal Axons of *Npc1*^{-/-} Cortical Neurons, Related to Figure 3F

Npc1^{-/-} cortical neurons cultured in microfluidic devices were incubated with MDW941 (100 nM) for 30 min to label active GCase-positive lysosomes before live imaging at DIV7-8. Time-lapse images were collected every 2 s for 90 frames totaling 3 min. Note that *Npc1*^{-/-} axons show reduced anterograde lysosome motility compared to control WT axons. Distal axon terminal is toward the right.

Video S5. Axonal Transport of Autophagic Vacuoles in Distal Axons of WT Cortical Neurons, Related to Figure S3G

WT cortical neurons cultured in microfluidic devices were transduced with a lentivirus encoding EGFP-LC3 at DIV0, followed by live imaging at DIV10. Time-lapse images were collected every 2 s for 90 frames totaling 3 min. Cell body is toward the left.

Video S6. Axonal Transport of Autophagic Vacuoles in Distal Axons of *Npc1*^{-/-} Cortical Neurons, Related to Figure S3G

Npc1^{-/-} cortical neurons cultured in microfluidic devices were transduced with a lentivirus encoding EGFP-LC3 at DIV0, followed by live imaging at DIV10. Time-lapse images were collected every 2 s for 90 frames totaling 3 min. Note that autophagic vacuoles in *Npc1*^{-/-} axons show similar motility patterns compared to control WT axons. Cell body is toward the left.

Video S7. Axonal Lysosome Delivery from the Soma to Distal Axons of *Npc1*^{-/-} Cortical Neurons, Related to Figure 7C

Npc1^{-/-} cortical neurons were cultured in microfluidic devices. At DIV8, MDW941 (5 nM) was applied only to the soma/dendritic chamber for 1 h to label active GCase-positive lysosomes in the cell body. Time-lapse images of axon bundles in microgrooves were collected every 1 s for 180 frames totaling 3 min. Axon chamber is toward the right.

Video S8. Axonal Lysosome Delivery from the Soma to Distal Axons of *Npc1*^{-/-} Cortical Neurons Treated with HPCD, Related to Figure 7C

Npc1^{-/-} cortical neurons cultured in microfluidic devices were treated with HPCD at 100 μ M for 48 h. At DIV8, MDW941 (5 nM) was applied only to the soma/dendritic chamber for 1 h to label active GCase-positive lysosomes in the cell body. Time-lapse images of axon bundles in microgrooves were collected every 1 s for 180 frames totaling 3 min. Axon chamber is toward the right.

REFERENCES

- Beard, H., Hassiotis, S., Gai, W.P., Parkinson-Lawrence, E., Hopwood, J.J., and Hemsley, K.M. (2017). Axonal dystrophy in the brain of mice with Sanfilippo syndrome. *Exp Neurol* 295, 243-255.
- Bilgin, M., Nylandsted, J., Jaattela, M., and Maeda, K. (2017). Quantitative Profiling of Lysosomal Lipidome by Shotgun Lipidomics. *Methods Mol Biol* 1594, 19-34.
- Boland, B., Kumar, A., Lee, S., Platt, F.M., Wegiel, J., Yu, W.H., and Nixon, R.A. (2008). Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease. *J Neurosci* 28, 6926-6937.
- Boland, B., and Platt, F.M. (2015). Bridging the age spectrum of neurodegenerative storage diseases. *Best Pract Res Clin Endocrinol Metab* 29, 127-143.
- Boland, B., Smith, D.A., Mooney, D., Jung, S.S., Walsh, D.M., and Platt, F.M. (2010). Macroautophagy is not directly involved in the metabolism of amyloid precursor protein. *J Biol Chem* 285, 37415-37426.
- Bolte, S., and Cordelieres, F.P. (2006). A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc* 224, 213-232.
- Bonifacino, J.S., and Neefjes, J. (2017). Moving and positioning the endolysosomal system. *Curr Opin Cell Biol* 47, 1-8.
- Bordi, M., Berg, M.J., Mohan, P.S., Peterhoff, C.M., Alldred, M.J., Che, S., Ginsberg, S.D., and Nixon, R.A. (2016). Autophagy flux in CA1 neurons of Alzheimer hippocampus: Increased induction overburdens failing lysosomes to propel neuritic dystrophy. *Autophagy* 12, 2467-2483.
- Cai, Q., Lu, L., Tian, J.H., Zhu, Y.B., Qiao, H., and Sheng, Z.H. (2010). Snapin-regulated late endosomal transport is critical for efficient autophagy-lysosomal function in neurons. *Neuron* 68, 73-86.
- Carstea, E.D., Morris, J.A., Coleman, K.G., Loftus, S.K., Zhang, D., Cummings, C., Gu, J., Rosenfeld, M.A., Pavan, W.J., Krizman, D.B., *et al.* (1997). Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis. *Science* 277, 228-231.
- Chen, F.W., Li, C., and Ioannou, Y.A. (2010). Cyclodextrin induces calcium-dependent lysosomal exocytosis. *PLoS One* 5, e15054.
- Chen, L., Wang, K., Long, A., Jia, L., Zhang, Y., Deng, H., Li, Y., Han, J., and Wang, Y. (2017). Fasting-induced hormonal regulation of lysosomal function. *Cell Res* 27, 748-763.
- Cheng, X.T., Xie, Y.X., Zhou, B., Huang, N., Farfel-Becker, T., and Sheng, Z.H. (2018). Characterization of LAMP1-labeled nondegradative lysosomal and endocytic compartments in neurons. *J Cell Biol* 217, 3127-3139.
- Cheng, X.T., Zhou, B., Lin, M.Y., Cai, Q., and Sheng, Z.H. (2015). Axonal autophagosomes recruit dynein for retrograde transport through fusion with late endosomes. *J Cell Biol* 209, 377-386.

Davidson, C.D., Ali, N.F., Micsenyi, M.C., Stephney, G., Renault, S., Dobrenis, K., Ory, D.S., Vanier, M.T., and Walkley, S.U. (2009). Chronic cyclodextrin treatment of murine Niemann-Pick C disease ameliorates neuronal cholesterol and glycosphingolipid storage and disease progression. *PLoS One* 4, e6951.

Diettrich, O., Mills, K., Johnson, A.W., Hasilik, A., and Winchester, B.G. (1998). Application of magnetic chromatography to the isolation of lysosomes from fibroblasts of patients with lysosomal storage disorders. *FEBS Lett* 441, 369-372.

Elrick, M.J., Yu, T., Chung, C., and Lieberman, A.P. (2012). Impaired proteolysis underlies autophagic dysfunction in Niemann-Pick type C disease. *Hum Mol Genet* 21, 4876-4887.

Elson, E.L., Fried, E., Dolbow, J.E., and Genin, G.M. (2010). Phase separation in biological membranes: integration of theory and experiment. *Annu Rev Biophys* 39, 207-226.

Epand, R.M. (2006). Cholesterol and the interaction of proteins with membrane domains. *Prog Lipid Res* 45, 279-294.

Farfel-Becker, T., Roney, J.C., Cheng, X.T., Li, S., Cuddy, S.R., and Sheng, Z.H. (2019). Neuronal Soma-Derived Degradative Lysosomes Are Continuously Delivered to Distal Axons to Maintain Local Degradation Capacity. *Cell Rep* 28, 51-64 e54.

Farias, G.G., Guardia, C.M., De Pace, R., Britt, D.J., and Bonifacino, J.S. (2017). BORC/kinesin-1 ensemble drives polarized transport of lysosomes into the axon. *Proceedings of the National Academy of Sciences of the United States of America* 114, E2955-E2964.

Ferguson, S.M. (2019). Neuronal lysosomes. *Neurosci Lett* 697, 1-9.

Filipek, P.A., de Araujo, M.E.G., Vogel, G.F., De Smet, C.H., Eberharter, D., Rebsamen, M., Rudashevskaya, E.L., Kremser, L., Yordanov, T., Tschakner, P., *et al.* (2017). LAMTOR/Ragulator is a negative regulator of Arl8b- and BORC-dependent late endosomal positioning. *J Cell Biol* 216, 4199-4215.

Fraldi, A., Annunziata, F., Lombardi, A., Kaiser, H.J., Medina, D.L., Spampanato, C., Fedele, A.O., Polishchuk, R., Sorrentino, N.C., Simons, K., *et al.* (2010). Lysosomal fusion and SNARE function are impaired by cholesterol accumulation in lysosomal storage disorders. *EMBO J* 29, 3607-3620.

Ganley, I.G., and Pfeffer, S.R. (2006). Cholesterol accumulation sequesters Rab9 and disrupts late endosome function in NPC1-deficient cells. *J Biol Chem* 281, 17890-17899.

Ge, Y., Gao, J., Jordan, R., and Naumann, C.A. (2018). Changes in Cholesterol Level Alter Integrin Sequestration in Raft-Mimicking Lipid Mixtures. *Biophys J* 114, 158-167.

Gowrishankar, S., Wu, Y., and Ferguson, S.M. (2017). Impaired JIP3-dependent axonal lysosome transport promotes amyloid plaque pathology. *J Cell Biol* 216, 3291-3305.

Gowrishankar, S., Yuan, P., Wu, Y., Schrag, M., Paradise, S., Grutzendler, J., De Camilli, P., and Ferguson, S.M. (2015). Massive accumulation of luminal protease-deficient axonal lysosomes at Alzheimer's disease amyloid plaques. *Proc Natl Acad Sci U S A* 112, E3699-3708.

Gurda, B.L., Bagel, J.H., Fisher, S.J., Schultz, M.L., Lieberman, A.P., Hand, P., Vite, C.H., and Swain, G.P. (2018). LC3 Immunostaining in the Inferior Olivary Nuclei of Cats With Niemann-Pick Disease Type C1 Is Associated With Patterned Purkinje Cell Loss. *J Neuropathol Exp Neurol* 77, 229-245.

Haidar, M., and Timmerman, V. (2017). Autophagy as an Emerging Common Pathomechanism in Inherited Peripheral Neuropathies. *Front Mol Neurosci* 10, 143.

Hirokawa, N., Niwa, S., and Tanaka, Y. (2010). Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease. *Neuron* 68, 610-638.

Hofmann, I., and Munro, S. (2006). An N-terminally acetylated Arf-like GTPase is localised to lysosomes and affects their motility. *J Cell Sci* 119, 1494-1503.

Hoque, S., Kondo, Y., Sakata, N., Yamada, Y., Fukaura, M., Higashi, T., Motoyama, K., Arima, H., Higaki, K., Hayashi, A., *et al.* (2020). Differential Effects of 2-Hydroxypropyl-Cyclodextrins on Lipid Accumulation in Npc1-Null Cells. *Int J Mol Sci* 21.

Jin, E.J., Kiral, F.R., Ozel, M.N., Burchardt, L.S., Osterland, M., Epstein, D., Wolfenberg, H., Prohaska, S., and Hiesinger, P.R. (2018). Live Observation of Two Parallel Membrane Degradation Pathways at Axon Terminals. *Curr Biol* 28, 1027-1038 e1024.

Kang, J.S., Tian, J.H., Pan, P.Y., Zald, P., Li, C., Deng, C., and Sheng, Z.H. (2008). Docking of axonal mitochondria by syntaphilin controls their mobility and affects short-term facilitation. *Cell* 132, 137-148.

Khobreakar, N.V., Quintremil, S., Dantas, T.J., and Vallee, R.B. (2020). The Dynein Adaptor RILP Controls Neuronal Autophagosome Biogenesis, Transport, and Clearance. *Dev Cell* 53, 141-153 e144.

Kilsdonk, E.P., Yancey, P.G., Stoudt, G.W., Bangerter, F.W., Johnson, W.J., Phillips, M.C., and Rothblat, G.H. (1995). Cellular cholesterol efflux mediated by cyclodextrins. *J Biol Chem* 270, 17250-17256.

Kimura, S., Noda, T., and Yoshimori, T. (2007). Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. *Autophagy* 3, 452-460.

Kimura, S., Noda, T., and Yoshimori, T. (2008). Dynein-dependent movement of autophagosomes mediates efficient encounters with lysosomes. *Cell Struct Funct* 33, 109-122.

Klionsky, D.J., Abdel-Aziz, A.K., Abdelfatah, S., Abdellatif, M., Abdoli, A., Abel, S., Abeliovich, H., Abildgaard, M.H., Abudu, Y.P., Acevedo-Arozena, A., *et al.* (2021). Guidelines for the use and interpretation of assays for monitoring autophagy (4th edition). *Autophagy*, 1-382.

Klionsky, D.J., and Emr, S.D. (2000). Autophagy as a regulated pathway of cellular degradation. *Science* 290, 1717-1721.

Ko, D.C., Milenkovic, L., Beier, S.M., Manuel, H., Buchanan, J., and Scott, M.P. (2005). Cell-autonomous death of cerebellar purkinje neurons with autophagy in Niemann-Pick type C disease. *PLoS Genet* 1, 81-95.

Lebrand, C., Corti, M., Goodson, H., Cosson, P., Cavalli, V., Mayran, N., Faure, J., and Gruenberg, J. (2002). Late endosome motility depends on lipids via the small GTPase Rab7. *EMBO J* 21, 1289-1300.

Lee, S., Sato, Y., and Nixon, R.A. (2011). Lysosomal proteolysis inhibition selectively disrupts axonal transport of degradative organelles and causes an Alzheimer's-like axonal dystrophy. *J Neurosci* 31, 7817-7830.

Levine, B., and Klionsky, D.J. (2004). Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell* 6, 463-477.

Lim, C.Y., Davis, O.B., Shin, H.R., Zhang, J., Berdan, C.A., Jiang, X., Counihan, J.L., Ory, D.S., Nomura, D.K., and Zoncu, R. (2019). ER-lysosome contacts enable cholesterol sensing by mTORC1 and drive aberrant growth signalling in Niemann-Pick type C. *Nat Cell Biol* 21, 1206-1218.

Lin, M.Y., Cheng, X.T., Tammineni, P., Xie, Y., Zhou, B., Cai, Q., and Sheng, Z.H. (2017). Releasing Syntaphilin Removes Stressed Mitochondria from Axons Independent of Mitophagy under Pathophysiological Conditions. *Neuron* 94, 595-610 e596.

Liu, B., Li, H., Repa, J.J., Turley, S.D., and Dietschy, J.M. (2008). Genetic variations and treatments that affect the lifespan of the NPC1 mouse. *J Lipid Res* 49, 663-669.

Liu, B., Turley, S.D., Burns, D.K., Miller, A.M., Repa, J.J., and Dietschy, J.M. (2009). Reversal of defective lysosomal transport in NPC disease ameliorates liver dysfunction and neurodegeneration in the npc1^{-/-} mouse. *Proc Natl Acad Sci U S A* 106, 2377-2382.

Lloyd-Evans, E., Morgan, A.J., He, X., Smith, D.A., Elliot-Smith, E., Sillence, D.J., Churchill, G.C., Schuchman, E.H., Galione, A., and Platt, F.M. (2008). Niemann-Pick disease type C1 is a sphingosine storage disease that causes deregulation of lysosomal calcium. *Nat Med* 14, 1247-1255.

Loftus, S.K., Morris, J.A., Carstea, E.D., Gu, J.Z., Cummings, C., Brown, A., Ellison, J., Ohno, K., Rosenfeld, M.A., Tagle, D.A., *et al.* (1997). Murine model of Niemann-Pick C disease: mutation in a cholesterol homeostasis gene. *Science* 277, 232-235.

Luzio, J.P., Pryor, P.R., and Bright, N.A. (2007). Lysosomes: fusion and function. *Nat Rev Mol Cell Biol* 8, 622-632.

Maday, S., and Holzbaur, E.L. (2012). Autophagosome assembly and cargo capture in the distal axon. *Autophagy* 8, 858-860.

Maday, S., and Holzbaur, E.L. (2016). Compartment-Specific Regulation of Autophagy in Primary Neurons. *J Neurosci* 36, 5933-5945.

Maday, S., Wallace, K.E., and Holzbaur, E.L. (2012). Autophagosomes initiate distally and mature during transport toward the cell soma in primary neurons. *J Cell Biol* 196, 407-417.

Maekawa, M., and Fairn, G.D. (2015). Complementary probes reveal that phosphatidylserine is required for the proper transbilayer distribution of cholesterol. *J Cell Sci* 128, 1422-1433.

Maetzel, D., Sarkar, S., Wang, H., Abi-Mosleh, L., Xu, P., Cheng, A.W., Gao, Q., Mitalipova, M., and Jaenisch, R. (2014). Genetic and chemical correction of cholesterol accumulation and impaired autophagy in hepatic and neural cells derived from Niemann-Pick Type C patient-specific iPS cells. *Stem Cell Reports* 2, 866-880.

Marques, A.R.A., and Saftig, P. (2019). Lysosomal storage disorders - challenges, concepts and avenues for therapy: beyond rare diseases. *J Cell Sci* 132.

Mellman, I. (1996). Endocytosis and molecular sorting. *Annu Rev Cell Dev Biol* 12, 575-625.

Mengel, E., Klunemann, H.H., Lourenco, C.M., Hendriksz, C.J., Sedel, F., Walterfang, M., and Kolb, S.A. (2013). Niemann-Pick disease type C symptomatology: an expert-based clinical description. *Orphanet J Rare Dis* 8, 166.

Micsenyi, M.C., Dobrenis, K., Stephney, G., Pickel, J., Vanier, M.T., Slaugenhaupt, S.A., and Walkley, S.U. (2009). Neuropathology of the Mcoln1(-/-) knockout mouse model of mucopolidosis type IV. *J Neuropathol Exp Neurol* 68, 125-135.

Mocholi, E., Dowling, S.D., Botbol, Y., Gruber, R.C., Ray, A.K., Vastert, S., Shafit-Zagardo, B., Coffer, P.J., and Macian, F. (2018). Autophagy Is a Tolerance-Avoidance Mechanism that Modulates TCR-Mediated Signaling and Cell Metabolism to Prevent Induction of T Cell Anergy. *Cell Rep* 24, 1136-1150.

Mukherjee, S., and Maxfield, F.R. (2004). Membrane domains. *Annu Rev Cell Dev Biol* 20, 839-866.

Naureckiene, S., Sleat, D.E., Lackland, H., Fensom, A., Vanier, M.T., Wattiaux, R., Jadot, M., and Lobel, P. (2000). Identification of HE1 as the second gene of Niemann-Pick C disease. *Science* 290, 2298-2301.

Ohara, S., Ukita, Y., Ninomiya, H., and Ohno, K. (2004). Axonal dystrophy of dorsal root ganglion sensory neurons in a mouse model of Niemann-Pick disease type C. *Exp Neurol* 187, 289-298.

Ohvo, H., and Slotte, J.P. (1996). Cyclodextrin-mediated removal of sterols from monolayers: effects of sterol structure and phospholipids on desorption rate. *Biochemistry* 35, 8018-8024.

Ory, D.S., Ottinger, E.A., Farhat, N.Y., King, K.A., Jiang, X., Weissfeld, L., Berry-Kravis, E., Davidson, C.D., Bianconi, S., Keener, L.A., *et al.* (2017). Intrathecal 2-hydroxypropyl-beta-cyclodextrin decreases neurological disease progression in Niemann-Pick disease, type C1: a non-randomised, open-label, phase 1-2 trial. *Lancet* 390, 1758-1768.

Palomo-Guerrero, M., Fado, R., Casas, M., Perez-Montero, M., Baena, M., Helmer, P.O., Dominguez, J.L., Roig, A., Serra, D., Hayen, H., *et al.* (2019). Sensing of nutrients by CPT1C regulates late endosome/lysosome anterograde transport and axon growth. *Elife* 8.

Peake, K.B., and Vance, J.E. (2012). Normalization of cholesterol homeostasis by 2-hydroxypropyl-beta-cyclodextrin in neurons and glia from Niemann-Pick C1 (NPC1)-deficient mice. *J Biol Chem* 287, 9290-9298.

Platt, F.M., d'Azzo, A., Davidson, B.L., Neufeld, E.F., and Tifft, C.J. (2018). Lysosomal storage diseases. *Nat Rev Dis Primers* 4, 27.

Pressey, S.N., Smith, D.A., Wong, A.M., Platt, F.M., and Cooper, J.D. (2012). Early glial activation, synaptic changes and axonal pathology in the thalamocortical system of Niemann-Pick type C1 mice. *Neurobiol Dis* 45, 1086-1100.

Pu, J., Guardia, C.M., Keren-Kaplan, T., and Bonifacino, J.S. (2016). Mechanisms and functions of lysosome positioning. *J Cell Sci* 129, 4329-4339.

Pu, J., Keren-Kaplan, T., and Bonifacino, J.S. (2017). A Ragulator-BORC interaction controls lysosome positioning in response to amino acid availability. *J Cell Biol* 216, 4183-4197.

Pu, J., Schindler, C., Jia, R., Jarnik, M., Backlund, P., and Bonifacino, J.S. (2015). BORC, a multisubunit complex that regulates lysosome positioning. *Dev Cell* 33, 176-188.

Rahbek-Clemmensen, T., Lycas, M.D., Erlendsson, S., Eriksen, J., Apuschkin, M., Vilhardt, F., Jorgensen, T.N., Hansen, F.H., and Gether, U. (2017). Super-resolution microscopy reveals functional organization of dopamine transporters into cholesterol and neuronal activity-dependent nanodomains. *Nat Commun* 8, 740.

Rocha, N., Kuijl, C., van der Kant, R., Janssen, L., Houben, D., Janssen, H., Zwart, W., and Neefjes, J. (2009). Cholesterol sensor ORP1L contacts the ER protein VAP to control Rab7-RILP-p150 Glued and late endosome positioning. *J Cell Biol* 185, 1209-1225.

Rosa-Ferreira, C., and Munro, S. (2011). Arl8 and SKIP act together to link lysosomes to kinesin-1. *Dev Cell* 21, 1171-1178.

Sardiello, M., Palmieri, M., di Ronza, A., Medina, D.L., Valenza, M., Gennarino, V.A., Di Malta, C., Donaudy, F., Embrione, V., Polishchuk, R.S., *et al.* (2009). A gene network regulating lysosomal biogenesis and function. *Science* 325, 473-477.

Sarkar, S., Carroll, B., Buganim, Y., Maetzel, D., Ng, A.H., Cassady, J.P., Cohen, M.A., Chakraborty, S., Wang, H., Spooner, E., *et al.* (2013). Impaired autophagy in the lipid-storage disorder Niemann-Pick type C1 disease. *Cell Rep* 5, 1302-1315.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., *et al.* (2012). Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9, 676-682.

Sharma, J., di Ronza, A., Lotfi, P., and Sardiello, M. (2018). Lysosomes and Brain Health. *Annu Rev Neurosci* 41, 255-276.

Tagliaferro, P., and Burke, R.E. (2016). Retrograde Axonal Degeneration in Parkinson Disease. *J Parkinsons Dis* 6, 1-15.

Tammineni, P., Ye, X., Feng, T., Aikal, D., and Cai, Q. (2017). Impaired retrograde transport of axonal autophagosomes contributes to autophagic stress in Alzheimer's disease neurons. *Elife* 6.

Taylor, A.M., Blurton-Jones, M., Rhee, S.W., Cribbs, D.H., Cotman, C.W., and Jeon, N.L. (2005). A microfluidic culture platform for CNS axonal injury, regeneration and transport. *Nat Methods* 2, 599-605.

Vanier, M.T. (2010). Niemann-Pick disease type C. *Orphanet J Rare Dis* 5, 16.

Walkley, S.U. (1998). Cellular pathology of lysosomal storage disorders. *Brain Pathol* 8, 175-193.

Walkley, S.U., Sikora, J., Micsenyi, M., Davidson, C., and Dobrenis, K. (2010). Lysosomal compromise and brain dysfunction: examining the role of neuroaxonal dystrophy. *Biochem Soc Trans* 38, 1436-1441.

Walkley, S.U., and Suzuki, K. (2004). Consequences of NPC1 and NPC2 loss of function in mammalian neurons. *Biochim Biophys Acta* 1685, 48-62.

Westbroek, W., Nguyen, M., Siebert, M., Lindstrom, T., Burnett, R.A., Aflaki, E., Jung, O., Tamargo, R., Rodriguez-Gil, J.L., Acosta, W., *et al.* (2016). A new glucocerebrosidase-deficient neuronal cell model provides a tool to probe pathophysiology and therapeutics for Gaucher disease. *Dis Model Mech* 9, 769-778.

Wilhelm, L.P., Wendling, C., Védie, B., Kobayashi, T., Chenard, M.P., Tomasetto, C., Drin, G., and Alpy, F. (2017). STARD3 mediates endoplasmic reticulum-to-endosome cholesterol transport at membrane contact sites. *EMBO J* 36, 1412-1433.

Willett, R., Martina, J.A., Zewe, J.P., Wills, R., Hammond, G.R.V., and Puertollano, R. (2017). TFEB regulates lysosomal positioning by modulating TMEM55B expression and JIP4 recruitment to lysosomes. *Nat Commun* 8, 1580.

Winkler, M.B.L., Kidmose, R.T., Szomek, M., Thaysen, K., Rawson, S., Muench, S.P., Wustner, D., and Pedersen, B.P. (2019). Structural Insight into Eukaryotic Sterol Transport through Niemann-Pick Type C Proteins. *Cell* 179, 485-497 e418.

Witte, M.D., Kallemeijn, W.W., Aten, J., Li, K.Y., Strijland, A., Donker-Koopman, W.E., van den Nieuwendijk, A.M., Bleijlevens, B., Kramer, G., Florea, B.I., *et al.* (2010). Ultrasensitive in situ visualization of active glucocerebrosidase molecules. *Nat Chem Biol* 6, 907-913.

Wong, Y.C., and Holzbaur, E.L. (2014). The regulation of autophagosome dynamics by huntingtin and HAP1 is disrupted by expression of mutant huntingtin, leading to defective cargo degradation. *J Neurosci* 34, 1293-1305.

Xie, Y., Zhou, B., Lin, M.Y., Wang, S., Foust, K.D., and Sheng, Z.H. (2015). Endolysosomal Deficits Augment Mitochondria Pathology in Spinal Motor Neurons of Asymptomatic fALS Mice. *Neuron* 87, 355-370.

Yang, D.S., Stavrides, P., Kumar, A., Jiang, Y., Mohan, P.S., Ohno, M., Dobrenis, K., Davidson, C.D., Saito, M., Pawlik, M., *et al.* (2017). Cyclodextrin has conflicting actions on autophagy flux in vivo in brains of normal and Alzheimer model mice. *Hum Mol Genet* 26, 843-859.

Yang, Y., Coleman, M., Zhang, L., Zheng, X., and Yue, Z. (2013). Autophagy in axonal and dendritic degeneration. *Trends Neurosci* 36, 418-428.

Yap, C.C., Digilio, L., McMahon, L.P., Garcia, A.D.R., and Winckler, B. (2018). Degradation of dendritic cargos requires Rab7-dependent transport to somatic lysosomes. *J Cell Biol* 217, 3141-3159.

Zhou, B., Yu, P., Lin, M.Y., Sun, T., Chen, Y., and Sheng, Z.H. (2016). Facilitation of axon regeneration by enhancing mitochondrial transport and rescuing energy deficits. *J Cell Biol* 214, 103-119.

Zidovetzki, R., and Levitan, I. (2007). Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. *Biochim Biophys Acta* 1768, 1311-1324.

Zigdon, H., Meshcheriakova, A., Farfel-Becker, T., Volpert, G., Sabanay, H., and Futerman, A.H. (2017). Altered lysosome distribution is an early neuropathological event in neurological forms of Gaucher disease. *FEBS Lett* 591, 774-783.