Glycolipid Trafficking in Drosophila Undergoes Pathway Switching in Response to Aberrant Cholesterol Levels

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In lipid storage diseases, the intracellular trafficking of sphingolipids is altered by conditions of aberrant cholesterol accumulation. Drosophila has been used recently to model lipid storage diseases, but the effects of sterol accumulation on sphingolipid trafficking are not known in the fly, and the trafficking of sphingolipids in general has not been studied in this model organism. Here, we examined the uptake and intracellular distribution of a fluorescent glycolipid analog, BODIPY-lactosyl-ceramide, in Drosophila neurons. The uptake mechanism and intracellular trafficking route of this simple glycolipid are largely conserved. Our principle finding is that cholesterol steers trafficking of the glycolipid between Golgi, lysosome, and recycling compartments. Our analyses support the idea that cholesterol storage in Drosophila triggers a switch in glycolipid trafficking from the biosynthetic to the degradative endolysosomal pathway, whereas cholesterol depletion eliminates recycling of the glycolipid. Unexpectedly, we observe a novel phenomenon we term “hijacking,” whereby lactosyl-ceramide diverts the trafficking pathway of an endocytic cargo, dextran, completely away from its lysosomal target. This work establishes that glycolipid trafficking in Drosophila undergoes changes similar to those seen in mammalian cells under conditions of cholesterol storage and therefore validates Drosophila as a suitable model organism in which to study lipid storage diseases.

INTRODUCTION

Drosophila has recently been adopted for the modeling of neurodegenerative lipid storage diseases, including Niemann-Pick, neuronal ceroid lipofuscinoses, and Tay-Sachs’s disease (Min and Benzer, 1997; Nakano et al., 2001; Huang et al., 2005; Mylllykangas et al., 2005; Phillips et al., 2008). The utility of the fly as a model in studying these diseases and examining their effects on lipid-trafficking routes will depend strongly on the development of lipid tracers that accurately report on the affected trafficking pathways. As in mammalian cellular models (Marks and Pagano, 2002), fluorescent glycosphingolipid analogs are potentially valuable tools in the characterization of fly models for lipid storage disorders and other diseases that affect lipid storage and metabolism, such as Alzheimer’s disease (Puglielli et al., 2003; Cutler et al., 2004; Wolozin, 2004; Maxfield and Tabas, 2005).

The aim of this study was to address two key issues, as a starting point for further studies using the Drosophila nervous system as a platform for studying lipid trafficking defects. First, we asked whether fluorescently tagged glycosphingolipids (GSLs), which have been used to study lipid trafficking in human cell types, are taken up and trafficked by Drosophila cultured neurons; and second, whether the trafficking of these GSLs is similarly affected by lipid perturbations (e.g., cholesterol storage) as their mammalian counterparts. Because these lipids are known to accumulate along with cholesterol in the endocytic compartments of Niemann-Pick diseased cells and in other lipid storage diseases, it is important to be able to follow their trafficking routes under normal and perturbed conditions such that one can better describe the cellular pathology and to evaluate the effectiveness of potential treatments.

To address the first question of how GSLs are trafficked in Drosophila, we use fluorescent dipyrromethene boron difluoride (BODIPY)-coupled analogs of a simple glycolipid, lactosyl-ceramide (lac-Cer), to show that a Drosophila neuronal cell line incorporates and traffics GSLs in a manner analogous to that observed in human cell lines. In this case, the glycosyl modification of the sphingolipid used (lac-Cer; galactos-β-glucose-β-ceramide) is structurally very similar to that found on an endogenous fly glycolipid, mactosyl-ceramide (mannose-β-glucose-β-ceramide). By carrying out time-course pulse-chase experiments with fluorescent BODIPY–lac-Cer on cells transfected with endolysosomal, Golgi, and recycling en-
dosomal markers, we establish that lac-Cer is less Golgi-localized than in mammalian fibroblasts and is found predominantly in recycling endosomes. Additionally, fluorescently tagged ceramide, which is thought to label exclusively Golgi in mammalian cells, traffics in part to endosomolysosomal vesicles.

In answer to the second aim of this study, whether sphingolipid trafficking in Drosophila is affected by cholesterol perturbations, we establish that cholesterol manipulations in neurons indeed influence the trafficking behavior of BODIPY–lac-Cer. After cholesterol overload, the effect is analogous to that which has been reported in the case of Niemann-Pick type C (NPC) fibroblasts, where lac-Cer accumulates aberrantly in an endosomolysosomal compartment (Pagano, 2003). Although mammalian studies were unable to assess changes in lac-Cer trafficking under cholesterol depletion, we find that cholesterol depletion causes an unexpectedly similar targeting of lac-Cer to endosomolysosomes, but also transiently increases it in Golgi.

Finally, we identify a novel phenomenon whereby fluorescent lac-Cer and an endocytic cargo dextran, which is normally targeted differently, influence each other’s behavior and traffic together when both are present simultaneously at the membrane. We propose to call this phenomenon “hijacking” because it involves the rerouting of the trafficking of one label by the other.

In summary, we have found that glycosphingolipid trafficking in Drosophila neurons follows routes similar to that described in mammalian cells and importantly, that its behavior is strongly influenced by conditions of cholesterol storage. These experiments constitute a first step in establishing a basis for studying the effects of lipid storage disorders and other neurodegenerative diseases on lipid trafficking.

**MATERIALS AND METHODS**

**Cell Culture and Transfection**

Neuronal Drosophila melanogaster (DL-DmBG2-c6) cells were obtained from the Drosophila Genome Resource Center (DGRC, Bloomington, IN) and cultured at 25°C in Schneider’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen), 0.125 IU/ml insulin (Biological Industries, Beit Haemek, Israel) and 1% antibiotic/antimycotic solution (Invitrogen). Transfections of LAMP-FL–cyclodextrin (M6), Q-GFP, and Rab11-GFP were carried out using CellFectin lipid reagent (Invitrogen) according to manufacturer’s instructions.

**Synthesis of BODIPY650/665–lac-Cer**

Far Red (FR) BODIPY650/665–lac-Cer was synthesized as follows:

A flame-dried 5-ml reaction vial was charged with 0.167 M of BODIPY-FL–lac-Cer (C5; Invitrogen) Rf 0.59; lac-Cer (C12) Rf 0.67. The relative fluorescence intensity of a fluorescent metabolized product, visible as a second spot below intact BODIPY FL–lac-Cer was used to calculate the percentage breakdown.

**TLC Analysis of Internalized BODIPY-lac-Cer**

C6 cells were incubated with 0.167 µM of BODIPY-FL–lac-Cer (C5; Invitrogen) for 30 min, washed, and further incubated in standard culture medium for 0, 6, and 14 h at 25°C. Lipids were extracted using standard protocols (Koval and Pagano, 1989) and run on TLC plates (Merck, Schwalbach, Germany) using the solvent methanol:chloroform:15 mM CaCl2 in the ratio of 60:58.5 (vol/vol). Plates were first imaged with a GFP filter using an Explore Optix MX system (GE Healthcare, Munich, Germany). They were later exposed to iodine crystals to calculate the RI (retention factor) values. Some standard lipids (Avanti Polar Lipids, Alabaster, AL) were also run on the TLC plates to estimate RI values; they are as follows: BODIPY FL–lac-Cer (C5; Invitrogen) RI = 0.59; lac-Cer (C12) RI = 0.57; Cer (C17) RI = 0.52; and GlcCer(C12) RI = 0.67. The relative fluorescence intensity of a fluorescent metabolized product, visible as a second spot below intact BODIPY FL–lac-Cer was used to calculate the percentage breakdown.

**Cholesterol Depletion and Overload**

For cholesterol depletion, mannosidaseII-GFP or LAMP-GFP-transfected cells were incubated with 10 mM methyl-β-cycloexetin (MβCD; Sigma-Aldrich, Munich, Germany) at 25°C for 30 min in serum-free medium, before labeling. After labeling, cells were further incubated in serum-free medium before cholesterol excess, 10 mM MβCD–cholesterol complexes were prepared as in Klein et al. (1995) and incubated with cells at 25°C for 30 min before labeling.

**Inhibitor Treatments**

Inhibitors used were as follows: Dynasore, Chlorpromazine, and nystatin (Sigma-Aldrich). Clostridium difficile type B toxin (Merck) was prepared according to manufacturer’s instructions, diluted in complete M3-BPYE cell culture medium, and applied at 25°C to cells.

C6 cells grown on eight-well glass chambers were treated with inhibitors as follows. Cells were treated with 25 µg/ml nystatin for 30 min, washed with HEPES/HBSS, and then pulse-labeled with fluorescent lipid in presence of nystatin. Samples were incubated with 5 µg/ml Chlorpromazine for 30 min and pulse-labeled with fluorescent lipid in presence of Chlorpromazine. Samples were incubated with 4 ng/ml C. difficile toxin B for 2 h, washed with HEPES/HBSS, and then pulse-labeled with fluorescent lipid in presence of the drug. Cells were incubated with 60 µM Dynasore for 30 min, washed with HEPES/HBSS, and pulse-labeled with fluorescent lipid in the presence of dynasore in complete M3-BPYE (Drosophila M3 medium with bacto-peptone and yeast extract).

**Microscopy and Postimaging Analysis**

Confocal microscopy was carried out using a Leica SP2 microscope with a 63X/1.4 NA oil water dipping lens (Heidelberg, Germany). For double-labeling experiments, control experiments were carried out using the single fluorophores, to verify that there was no cross-talk between the emission channels. For colocalization experiments all images in a given experiment were taken and processed identically. Colocalization values were calculated for at least 15 cells at each time point, using the Costes algorithm (Costes et al., 2004) that is available through ImageJ (rsb.info.nih.gov/ij) with the additional plugins: “Colocalization Test” and “Colocalization Threshold” by T. Collins and W.
LacCer Is Taken Up in an Endocytic Pathway by Drosophila Neurons

We studied the uptake and trafficking of fluorescently labeled analogs of the simple GSL lac-Cer in the Drosophila larval brain-derived neuronal cell line DL-DmBG2-c6 (hereafter referred to as c6) (Ui et al., 1994). Flies produce GSLs, but because their content and structure are different from mammalian GSLs (Seppo et al., 2000), it was important to establish that mammalian-type GSLs could be incorporated, as they are in mammalian cells and that flies traffic these lipids in an analogous way. Fluorescent lac-Cer trafficking has been characterized as an indicator of GSL trafficking defects in human fibroblasts where cholesterol trafficking is disturbed (Puri et al., 2001; Choudhury et al., 2002, 2004; Sharma et al., 2003; Singh et al., 2003), and in fact, these two lipid classes are known to traffic interdependently (Silence et al., 2002; Puri et al., 2003; Mukherjee and Maxfield, 2004; Sharma et al., 2004; te Vruchte et al., 2004).

As is the case in mammalian cells, exogenously applied analogs are taken up via the plasma membrane into endocytic vesicles by Drosophila c6 neurons (Figure 1A and B). Fluorescent BODIPY-FL–tagged lac-Cer (BODIPY-FL–lac-Cer; Invitrogen) gradually becomes incorporated into mobile endocytic vesicles after 30-min incubation on the cells (shown after 1-h chase, Supplemental Video 1; video1.mp4) over ~10 min, from an initially uniform distribution at the plasma membrane. This behavior was observed for both BODIPY-FL–tagged lac-Cer, as well as a novel BODIPY-FL–tagged CR Ceramide (red) also colocalizes to a limited extent with the lysosomal markers dextran-Alexa488 (green) and Lysotracker-green that were incubated on the cells 3 h into the chase and imaged after 2 additional hours of chase (total chase time for TR-ceramide was not quantified).

Results

The uptake of the following analogs of the simple GSL lac-Cer was studied: BODIPY-FL–lac-Cer, BODIPY-505–510 FL–tagged lac-Cer, as well as BODIPY-FL–tagged cholesteryl sulfate and cholesteryl esters. The uptake of these analogs was compared with uptake of the simple GSL lac-Cer, which was tagged with the fluorescent lipid BODIPY-FL (green). The uptake of these analogs was studied in primary cultured Drosophila neurons (Figure 1). BODIPY-FL–tagged lac-Cer was added to primary cultured Drosophila neurons for 5 min and chased for 15 h to label late endosomes and lysosomes. Note that colocalization with dextran (red trace) is not disturbed (Puri et al., 2001; Choudhury et al., 2002, 2004; Sharma et al., 2003; Singh et al., 2003), and in fact, these two lipid classes are known to traffic interdependently (Silence et al., 2002; Puri et al., 2003; Mukherjee and Maxfield, 2004; Sharma et al., 2004; te Vruchte et al., 2004).

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tagged lac-Cer (see Materials and Methods), and these two analogs colocalized nearly completely (Figure 1A), indicating that the choice of fluorophore does not influence the uptake or subcellular trafficking of lac-Cer.

BODIPY-FR–lac-Cer colocalizes with LAMP-GFP (Pulipparacharuvil et al., 2005) at a peak value of tM lac-Cer/H11015 30% after 30 min of chase time (Figure 1C); colocalization was calculated using the Manders algorithm with Costes thresholding (Manders et al., 1993; Costes et al., 2004). At this time, a fluid-phase marker of endocytic uptake, 10-kDa dextran, passes through the sorting endosomal compartment (Sriram et al., 2003). LAMP-GFP, although ostensibly a lysosomal marker, is also partially distributed in earlier, highly mobile endosomal compartments (R.K., unpublished observations), some of which may be mildly acidified (pH 5.9–6) sorting endosomes (Hunziker and Geuze, 1996; Steinert et al., 2008). After the 30-min peak, colocalization with LAMP-GFP fell over the next hour to ~15%, when dextran at a similar time would be exiting the sorting endosome and entering the late endosomal portion of the pathway (Sriram et al., 2003). Thereafter, colocalization remained constant at ~15% for up to 4 h of chase (Figure 1, B and C). Therefore, we conclude that BODIPY–lac-Cer transiently passes through sorting endosomes at ~30 min en route to other compartments.

As a comparison to the colocalization of BODIPY-FR–lac-Cer with LAMP-GFP, dextran-Alexa594 was chased in the cells overnight to label late endosomes/lysosomes, and these cells were then labeled with BODIPY-FR–lac-Cer and imaged after the given chase times (Figure 1C), Initial colocalization is lower than with LAMP-GFP, and the peak in presumptive sorting endosomes is absent (compare red dextran trace to black LAMP-GFP trace) but increases to ~20% after 90 min.

We also observed that a nonglycosylated fluorescent sphingolipid, FL-BODIPY- or TR-BODIPY-ceramide (FL- or TR-Cer), is taken up into mobile endosomes as is the glycosylated BODIPY–lac-Cer and exhibits the interesting property of trafficking in tubulovesicular compartments that move along the length of neuronal processes (see Supplemental Video 2). TR-Cer also showed some colocalization with dextran-Alexa488 and lysotracker-green, which were added after the TR-cer labeling, although this was not quantified (Figure 1D).

**BODIPY–lac-Cer Uptake Is Sensitive to Inhibitors of Cholesterol- and Dynamin-mediated Endocytosis**

To discover the mechanism of BODIPY–lac-Cer uptake into Drosophila neurons, cells were treated with pharmacological inhibitors of different uptake mediators, then incubated with BODIPY–lac-Cer or control labels, and chased in the presence of the drug. Figure 2 shows an uptake time course, where the number of internalized vesicles containing...
BODIPY–lac-Cer is quantified (see Materials and Methods) at different times after chase. Inhibition of clathrin-mediated uptake by chlorpromazine (Figure 2, A and F) also inhibits BODIPY–lac-Cer uptake substantially, but this recovers to some extent after 1 h. FM1-43 uptake is not strongly affected (Figure 2A).

To distinguish between rho-family GTPase-mediated uptake versus dynamin-mediated uptake of BODIPY–lac-Cer in fly neurons, the rho-family GTPase inhibitor Clostridium type B toxin and the dynamin inhibitor Dynasore (Macia et al., 2006) were applied. Inhibition of rho-mediated uptake by Clostridium B toxin (Puri et al., 2001), similar to clathrin uptake, lowered BODIPY–lac-Cer uptake initially (Figure 2B), but this also recovered to nearly normal levels after 60 min. As expected, uptake of dextran-Alexa594, which in mammalian cells is mediated by rho family member cdc42, was strongly inhibited compared with the control (Figure 2B). Dynasore inhibited uptake of BODIPY–lac-Cer throughout the time course (Figure 2, C and F).

Finally, nystatin was used as an inhibitor of raft-mediated uptake by virtue of its cholesterol-extracting properties (Puri et al., 2001). This was as effective as Dynasore in continuously inhibiting BODIPY–lac-Cer uptake throughout the time course and also inhibited CtxB uptake compared with control (Figure 2, D–F). Apparently unlike mammalian fibroblasts, however, where lac-Cer uptake is completely inhibited by raft- and dynamin inhibitors, but completely unaffected by clathrin inhibitors (Puri et al., 2001), the inhibition of fluorescent lac-Cer uptake in fly neurons is only partial.

The inhibitor studies in summary suggest that although BODIPY–lac-Cer uptake can be perturbed by interference with several different uptake pathways (including clathrin- and rho-family mediated), the effect of dynamin inhibition is stronger than that of the rho family inhibitor, similar to the case in mammalian cells (Puri et al., 2001). As in mammalian cells, nystatin also effectively inhibited uptake, indicating that a cholesterol-mediated mechanism, and therefore a possible association with lipid rafts, is at least in part responsible for uptake.

**Lac Cer Is Only Partially Golgi Associated**

Because BODIPY–lac-Cer had been characterized as traversing the Golgi in human fibroblasts, we tested for colocalization between BODIPY–lac-Cer and the transfected Drosophila Golgi marker MannII-GFP (Bard et al., 2006; Figure 3, A and B). Starting from <10% colocalization at 30 min, colocalization increased to 20% at 1 h, and thereafter hovered near 20% for up to 4 h (Figure 3B). This is significantly lower than would be expected for an exclusively Golgi marker. To eliminate the possibility that some of the fluorescent lac-Cer was being metabolized into other products, which might localize differently, the labeled cells were extracted, and the lipids were analyzed by TLC. After 6 h of chase, all of the BODIPY–lac-Cer was still present (see Materials and Methods and Supplementary Figure S3). Only after 14 h of incubation, fluorescence intensity quantification of the two spots indicated that an average of 16.5% of BODIPY–lac-Cer had been metabolized to other products.

Previously, it was reported that lac-Cer is partially trafficked to the Golgi compartment, with a minor early contribution from recycling endosomes, which cycle the lipid back to the plasma membrane (Sharma et al., 2003). Given the relatively low degree of BODIPY–lac-Cer trafficking to the...
Golgi and endolysosomal compartments in our cell line (each only ~20%; Figures 1, B and C, and 3, A and B), we tested for its presence in other compartments. We found that a substantial portion (35–40%) of BODIPY–lac-Cer trafficked to rab11-GFP–labeled recycling endosomes (the rab11-GFP plasmid was a kind gift of H. Chang [Purdue University]; Figure 3, C and D). Sharma et al. (2003) also report a contribution from the recycling endosome to BODIPY–lac-Cer’s distribution in fibroblasts, but in their report this dropped rapidly (after 15 min) because the label was recycled back to the plasma membrane. BODIPY–lac-Cer in the fly cells trafficked primarily to recycling endosomes, and about half as much was found in Golgi over an extended time period, but no major loss of the label was noted.

From our quantitative time-course colocalization studies, it appears that after passage through sorting endosomes, BODIPY–lac-Cer is present primarily in the recycling endosomal compartment, and the remainder is localized to the Golgi and to lysosomes. A small proportion remains in LAMP-GFP–positive vesicles that we presume to be sorting endosomes. This is similar to what was reported in fibroblasts (Puri et al., 2001; Sharma et al., 2003), but our study’s findings differ in that the Golgi contribution is much less than that of recycling endosomes.

**BODIPY–lac-Cer Can Hijack the Trafficking Route of an Endocytic Cargo**

Early studies by Pagano and coworkers showed that lac-Cer stimulates caveolar uptake from the plasma membrane (Sharma et al., 2004). Our experiments reveal a novel effect, distinct from simple stimulation of uptake, whereby lac-Cer and another endocytic cargo, dextran, exert a mutual influence on each other’s trafficking routes. Rerouting through a novel pathway would not be expected if lac-Cer were merely stimulating endocytosis, and carried dextran with it.

In mammalian cells, lac-Cer and dextran are taken up via different endocytic pathways that depend on different accessory proteins (dynamin vs. cdc42), and are inhibited by different drugs (Puri et al., 2001; Sharma et al., 2003; Singh et al., 2003). Similarly, in Drosophila cells BODIPY–lac-Cer and dextran trafficking is almost entirely separate when incubated independently (Figure 4), but surprisingly, they show almost complete colocalization when incubated directly one after the other.

Sequential labeling of BODIPY-FL–lac-Cer and then FR-dextran (lac-Cer pulse, 3-h chase, dextran pulse, 2-h chase) or conversely, dextran and then BODIPY-FL–lac-Cer (dextran pulse, 2-h chase, lac-Cer pulse, 3-h chase) both resulted in nearly no colocalization (Figure 4, B and D). This was expected, because after 5 h BODIPY lac-Cer is not targeted primarily to lysosomes (see Figure 1; Puri et al., 2001), whereas dextran is predominantly lysosomal. Unexpectedly, however, BODIPY–lac-Cer and dextran traffic completely together when added simultaneously (the thresholded Manders’ coefficient $tM \approx 80\%$; Figure 4, A and C). This is not likely to be due to direct binding between the two labels, because the BODIPY–lac-Cer is washed out briefly before addition of the dextran, and the same effect was seen when the relative amounts of the two labels were varied (Figure 4E). The effect of BODIPY–lac-Cer on the trafficking of other cargoes appears not to be limited to insect cells, because a similar effect was observed in mammalian SH-SY5Y neuroblastoma (Figure S4), where dextran incubated with BODIPY–lac-Cer simultaneously colocalized to a greater extent than when the two were incubated sequentially and chased for long enough to reach their final destinations. $tM_{\text{dextran}} = 0.4 \pm 0.021$ when incubated sequentially, versus $0.67 \pm 0.015$ when incubated simultaneously ($p = 3.24226 \times 10^{-11}$).

We wanted to find out more about the mechanism of this rerouting phenomenon and whether the new trafficking route was more like BODIPY–lac-Cer, or dextran, or a hybrid. To characterize the new routes of the BODIPY–lac-Cer + dextran combination in Drosophila neurons, we carried out time-course colocalizations with BODIPY–lac-Cer + dextran in the presence of either a Golgi or a lysosomal reference marker and calculated colocalization of total BODIPY–lac-Cer intensity with the reference marker ($tM_{\text{lac-Cer}}$, Figure 5).

In each case, BODIPY–lac-Cer colocalized completely with pulsed dextran (as seen in Figure 4A), but comparison with LAMP-GFP showed that BODIPY–lac-Cer diverted dextran away from endolysosomes (cf. green line with dashed black line; Figure 5A). Comparison with dextran incubated overnight to label lysosomes showed an even stronger diversion of the pulsed dextran away from lysosomes (compare green line with dashed black line; Figure 5B). The difference between the colocalization curves with LAMP-GFP versus overnight dextran is not unexpected, because LAMP-GFP is not strictly confined to lysosomes, as discussed earlier, and these two ostensibly lysosomal labels colocalize only ~40% with each other (Figure 4C).

BODIPY–lac-Cer’s own itinerary is also slightly altered in the presence of dextran (cf. red lines with dashed black lines in Figure 5, A–C). In particular, BODIPY–lac-Cer + dextran follows a slower route toward the Golgi than BODIPY–lac-Cer alone (Figure 5C). BODIPY–lac-Cer + dextran appears to arrive late and persist at a slightly higher than normal level in endolysosomes labeled with LAMP-GFP (Figure 5A), whereas it is suppressed in lysosomes labeled with dextran overnight (Figure 5B). However, these changes in BODIPY–lac-Cer’s trafficking behavior are not as drastic as those in dextran’s pathway.

We conclude that BODIPY–lac-Cer interacting with a ligand may direct that ligand to an alternate intracellular path. Specifically, dextran, which ordinarily follows an endosome →late endosome →lysosome path, is rerouted completely away from late endolysosomes when BODIPY–lac-Cer is also present. We propose to call this phenomenon hijacking because it involves the rerouting of the trafficking of one agent by the other.

To test whether the uptake mechanism of the dextran is altered by BODIPY–lac-Cer as well as its trafficking route, we treated cells with inhibitors of dextran’s and lac-Cer’s uptake, as in Figure 2, and compared the degree of inhibition in the presence or absence of the other label (Figure 5, D–F). The colocalization between BODIPY–lac-Cer and dextran added together in the presence of inhibitors was also measured and was found to be still much higher than normal (i.e., sequential), although not as high as without the inhibitors (Figure 5D). This shows that even in the presence of one inhibitor or the other, the uptake of BODIPY–lac-Cer and dextran do not completely uncouple when they are both present at the membrane simultaneously. Images of cells incubated with both labels and the inhibitors are shown in Figure 5F.

*Clostridium* B toxin treatment of BODIPY–lac-Cer + dextran together showed that the uptake pathway of dextran is altered by the presence of the BODIPY–lac-Cer, because the inhibition is not as severe when BODIPY–lac-Cer is there (Figure 5E, left, dextran graph). In contrast, dynasore’s effect on BODIPY–lac-Cer is just as inhibitory whether or not dextran is present (Figure 5E, right, BODIPY–lac-Cer graph).

Together, these experiments suggest that the uptake mechanism of dextran can be altered to a certain extent by lac-Cer present simultaneously at the membrane and that
the subsequent trafficking of dextran is strongly shifted by lac-Cer toward a nonlysosomal target.

**BODIPY–lac-Cer Trafficking to Golgi versus Lysosomes Is Controlled by a Cholesterol Switch**

Pagano and colleagues have characterized changes in GSL trafficking in human fibroblasts as a cell culture model for lipid storage diseases, in particular NPC (Pagano, 2003). Under conditions of aberrant endolysosomal cholesterol storage brought about by the disease, BODIPY–lac-Cer accumulated in late endolysosomal compartments and was diverted from its normal Golgi itinerary (Choudhury et al., 2002, 2004). Similarly, aberrant trafficking of GSL to lysosomes was found in a model of Gaucher disease (Sillence et al., 2002). Therefore, it was important for us to address whether trafficking of GSLs in fly neurons is affected similarly to that in diseased human cells, under analogous conditions of lipid storage.

When cells are overloaded with cholesterol (see Materials and Methods), trafficking to endolysosomes is strongly increased at the expense of Golgi-directed trafficking (cf. blue trace to black trace, Figure 6, A and D). The initial uptake pathway, presumably through early and sorting endosomes, is not perturbed (Figure 6D, up to 60 min), but becomes noncoincident at the point of divergence between the Golgi and endolysosomal pathways (60–90 min).
Figure 5. BODIPY–lac-Cer uptake in the presence of dextran alters trafficking itineraries of both tracers. Endolysosomes, Golgi, and lysosomes were labeled with LAMP-GFP (A), Alexa546-dextran (B), and MannII-GFP, chased for 15 h (C), and subsequently labeled with BODIPY-FL–lac-Cer and Alexa647-dextran simultaneously to determine whether the trafficking route of either label changed in the presence of the other. (A) After simultaneous incubation, both labels accumulate slowly in LAMP-positive compartments (black trace), more than would be expected for BODIPY–lac-Cer (red line), but less than dextran by itself (green line). (B) Trafficking of FR-dextran to lysosomes labeled by 15-h incubation of Alexa546-dextran (green line) was completely inhibited in the presence of BODIPY–lac-Cer (black trace). (C) Trafficking of BODIPY–lac-Cer to Golgi (normally ~20%; red line) is also inhibited by the presence of dextran (black trace). dextran does not normally traverse the Golgi in the absence of BODIPY–lac-Cer (green line). Significant differences in colocalization at given time points; *p < 0.05. (D) Quantification of colocalization of BODIPY-FR–lac-Cer with dextran added simultaneously, in the presence or absence of the inhibitor Clostridium toxin B (black), which affects dextran uptake (see Figure 1), and dynasore (light gray), which affects BODIPY–lac-Cer uptake. Colocalization remains nearly as high as after the control simultaneous incubation (dark gray) and much higher than after sequential incubation (hatched). (E, left) Quantification of dextran-Alexa488 uptake efficiency after 90 min, either alone (white) or together with BODIPY-FR–lac-Cer (black) in untreated cells, versus lower uptake in Clostridium toxin B–treated cells (gray). Uptake inhibition of dextran is rescued to an intermediate level by the presence of BODIPY–lac-Cer (dark gray hatched). Uptake of dextran alone is not
Under conditions of cholesterol depletion by incubation with MβCD, the converse effect is observed, with respect to Golgi trafficking: BODIPY–lac-Cer trafficking to Golgi appears to be initially slower than normal, until 2 h, and then shows an enhanced peak at 3 h (Figure 6A), which returns to a similar baseline as in the control cells after extended chase (4 h). The effectiveness of the cholesterol depletion and overload treatments were tested by quantifying sterol levels using the Amplex Red assay (Invitrogen), which showed that extracts of MβCD-treated c6 neurons had a 47 ± 8% reduction of cholesterol, and cholesterol-overloaded cells showed an increase of 250 ± 13% (Hebbbar et al., 2008). In Figure 9 of Steinert et al. (2008) we also showed that cholesterol-loaded c6 neurons labeled much more strongly with filipin, confirming the presence of excess free cholesterol. Here, representative images of cells that were treated and labeled with the different markers and BODIPY–lac-Cer (Figure 6, B–I) suggest that the observed changes in BODIPY–lac-Cer trafficking were not a result of less effective uptake due to cholesterol depletion or overload. However, the morphology of the endolysosomal compartments did seem to be altered under cholesterol loading (e.g., Figure 6F).

We were surprised to find that cholesterol depletion increased BODIPY–lac-Cer trafficking to endolysosomes to a similar degree as cholesterol excess late in the pathway (after ~90 min), with total colocalization for both being around 50% after 3 h, versus only 15% in untreated controls (Figure 6D). Colocalization of BODIPY–lac-Cer with the recycling compartment labeled with rab11-GFP was also monitored under both cholesterol excess and depletion, to find out whether the ectopic BODIPY–lac-Cer found especially in endolysosomes under both these conditions was being diverted from recycling endosomes (Figure 6G). Ordinarily, a large portion (~40%) of BODIPY–lac-Cer targets to the recycling compartment. Under cholesterol depletion, trafficking to recycling endosomes was almost eradicated, presumably going instead to sorting and then Golgi vesicles, in turn (red trace, cf. Figure 6, G with A and D; summarized schematically in Figure 7). It was also reduced gradually by ~10–15% under cholesterol excess, perhaps due to the slower accumulation of BODIPY–lac-Cer in late endolysosomes with cholesterol excess (cf. blue trace, Figure 6G,D; indicated by a dotted arrow in Figure 7).

To demonstrate the applicability of this method to actual Drosophila genetic models, we applied the same probes, BODIPY–lac-Cer and dextran, to primary cultured neurons from third instar larval brains of different control strains, versus genetic conditions that might be expected to alter lipid metabolism and/or storage. We tested mutants in the AMP-activated protein kinase (AMPK) gamma subunit l öchrig, which are neurodegenerative and accumulate abnormally high levels of free sterol in the brain (Tschiapé et al., 2002), as well as animals overexpressing the presumptive lysosomal/autophagic trafficking protein Blue cheese (Finley et al., 2003; Simonsen et al., 2007; Lim and Kraut, 2009) in the nervous system. Overexpression of Blue cheese in neurons results in abnormally high levels of sterol-ester and higher sterol levels overall in the adult brain (our unpublished observations). BODIPY–lac-Cer was found to be taken up readily by these neurons, and the degree of colocalization of BODIPY–lac-Cer with fluorescent dextran-Alexa488 that had been pulsed and chased overnight was measured after live pulse-chase experiments similar to those described in Figure 6 (see Materials and Methods). A comparison of neurons from larval brains of yw and elav-GAL4 strains as controls, with neurons from l öchrig and elav> blue cheese brains suggested that there may be slightly greater trafficking of the sphingolipid analogue to degradative compartments in these neurodegenerative situations, although colocalization levels in general were higher than in the c6 cell line. Colocalization of BODIPY–lac-Cer with dextran-Alexa488 in the control neurons ranged from about tM lac-Cer ~ 0.51 to ~0.63. Primary neurons from l öchrig and elav>EP-blue cheese animals had higher colocalization levels than yw (tM lac-Cer 0.68 and 0.70, vs. 0.51 respectively), but the differences between these and the elav-GAL4 controls were not as great (Figure S5). Altering lipid conditions in the culture medium and higher-resolution imaging methods will be tested as possible approaches to examine more closely what are now detectable as only subtle trafficking differences.

**DISCUSSION**

In this report, we have carried out the first characterization of the intracellular trafficking behavior of a simple glycolipid, lac-Cer, in Drosophila cells. Using systematic, quantitative time-course colocalizations and pharmacological inhibition, we have shown that a fluorescent glycolipid analog, BODIPY–FR–lac-Cer, is endocytosed primarily by a cholesterol- and dynamin-mediated mechanism and traffics between the biosynthetic (Golgi) and degradative (endolysosomal) pathways, with a major component (~40%) traveling through recycling endosomes. The trafficking switch between biosynthetic and degradative pathways appears to depend on free cholesterol levels in a manner similar to what was seen in mammalian cells (Puri et al., 2001).

Because the composition of Drosophila sphingolipids and glycolipids is somewhat different from those of mammals, it was not obvious that this sphingolipid would behave in an analogous manner to that in the mammalian system. Fly sphingolipids, as well as their membrane lipids in general, have shorter acyl chains (a C14 sphingoid chain instead of the more common C18 in mammals; Holthuis et al., 2001). Glycosphingolipids in fly contain mannose not galactose as the second sugar moiety (Wiegand, 1992) and are not sialylated but instead contain phosphoethanolamine substituted N-acetyl-glucosamine (GlcNAc; Seppo et al., 2000). In spite of these differences, our findings rather surprisingly support an overall conservation of the behavior of at least one simple two-sugar glycosphingolipid, lac-Cer. This may be because of the structural similarity between lac-Cer and the Drosophila equivalent mactosyl-cer, which besides the acyl chain length differs only in the orientation of one hydroxyl group on the terminal hexose (Seppo et al., 2000).

**The Intracellular Pathway and Sterol-dependent Uptake of lac-Cer in Drosophila is Similar to Mammalian Cells**

Although the overall intracellular distribution BODIPY–lac-Cer was conserved in Drosophila neurons, we found a stronger contribution from the recycling and lysosomal compartments than from the Golgi compared with what was previ-

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**Figure 5 (cont).** significantly inhibited by dynasore (light gray). (E, right) Quantification of BODIPY–FR–lac-Cer uptake efficiency after 90 min, either alone in untreated cells (white) or together with dextran (black), versus lower uptake in dynasore-treated cells (gray). Uptake inhibition is not rescued by the presence of dextran (dark gray hatched), in contrast to the situation on the left. Uptake of BODIPY–FR–lac-Cer alone is not inhibited by *Clostridium* toxin B (light gray). (F) Images of cells showing that the presence of inhibitors does not prevent colocalization of the two labels: BODIPY–FR–lac-Cer (red) and dextran (green).
Figure 6. BODIPY–lac-Cer trafficking between Golgi, recycling endosomes, and the endolysosomal pathway is controlled by a cholesterol switch in fly neurons. (A, D, and G) Cholesterol depletion (red trace in all graphs). Loss of cholesterol leads to increased traffic through a LAMP-positive sorting compartment at 60 min (D) and then a significant but transient accumulation in Golgi labeled by MannII-GFP (A) at 180 min. BODIPY–lac-Cer finally accumulates in LAMP-positive late endolysosomes. Passage of BODIPY–lac-Cer through recycling endosomes in contrast is much reduced (G). The drop in Golgi at 240 min is probably not due to production of noncolocalizing metabolites of BODIPY-lac-Cer, because labeled lac-Cer remains stable for at least 6 h (see Supplemental Figure 3). Cholesterol excess (blue traces in all graphs): Cholesterol overloading strongly suppresses Golgi-targeting (A), in contrast to the enhancement of Golgi targeting seen under cholesterol depletion (red trace, A), and leads to aberrant accumulation in enlarged LAMP-positive (D) compartments, but with a later time course than depletion (compare blue and red traces, D). Colocalization is expressed as tMlac-Cer, converted to %. Significance of the differences in colocalization: *p < 0.05. (B, C, E, F, H, and I) Images of c6 neurons transfected with markers of the different compartments and depleted or loaded with cholesterol: Golgi (MannII-GFP in B and C), endolysosomes (LAMP-GFP in E and F), and recycling endosomes (rab11-GFP in H and I; all green), and labeled with BODIPY-FR–lac-Cer (red). Cholesterol depletion and excess are shown for each compartment. LAMP-GFP–positive endolysosomes are visibly enlarged under cholesterol excess (cf. E and F) and FR–lac-Cer–carrying vesicles appear more diffuse (C, F, and I). Bars, 4 μm.
lac-Cer may be transported out of the Golgi to endolysosomes. Under cholesterol overload (right), the Golgi compartment is completely bypassed, whereas endo/lysosomes receive much more than normal BODIPY–lac-Cer and recycling endosomes somewhat less. Question mark indicates that BODIPY–lac-Cer may be transported from recycling to endolysosomes. In summary, abnormally low amounts of sterol in *Drosophila* neurons seem to favor a Golgi and lysosomal pathway at the expense of recycling, whereas excess sterol favors a lysosomal pathway mainly at the expense of Golgi.

The most effective and long-lasting interference with uptake of BODIPY–lac-Cer in fly neurons was obtained with inhibitors of raft-mediated endocytosis and dynamin, but the clathrin inhibitor chlorpromazine also inhibited uptake nearly as strongly. The effects exerted by the rho GTPase inhibitor were only transient, by contrast. Similarly, lac-Cer in mammalian cells was shown by Pagano’s group to be taken up by a dynamin- and cholesterol-dependent (presumably raft-mediated) pathway (Puri et al., 2001) independent of clathrin.

The partial inhibition by the clathrin inhibitor was unexpected, given the results in mammalian cells. As a possible explanation for this, it should be noted that chlorpromazine inhibited the uptake of another presumptive raft marker CtxB to a significant extent (Broeck et al., 2007), and multiple uptake mechanisms are known to operate for several other agents that are taken up by lipid rafts (Massol et al., 2004; Deinhardt et al., 2006).

The uptake of a glycolipid by an apparently cholesterol-mediated mechanism similar to that in mammalian cells is an important finding, given that flies are cholesterol auxotrophs and have comparatively low membrane sterol content (Marheineke et al., 1998). This has led to some uncertainty as to the role of cholesterol in fly cell biology, especially with regard to its possible function in endo-lysosomal trafficking and lipid homeostasis versus a more predominant role in ecdysone hormone production (Huang et al., 2005; Fluegel et al., 2006; Baker and Thummel, 2007; Huang et al., 2007). Although the precise roles of sterols in the fly are still unclear, lipid rafts in the form of detergent-resistant membranes rich in sphingolipids and sterols can be biochemically isolated from fly tissues, and the association of raft-localized markers can be disrupted by cholesterol extraction, as with mammalian membranes (Rietveld et al., 1999; Zhai et al., 2004; Hebar et al., 2008).

Another interesting outcome of this study was the identification of a phenomenon we call hijacking, which refers to the observation that BODIPY–lac-Cer can influence the trafficking of a second cargo, directing both to a novel intracellular location. This is distinct from the report of Pagano and coworkers that caveolar uptake was stimulated by lac-Cer (Sharma et al., 2004), because it involves redirection of trafficking, in this case of dextran away from the endolysosomal pathway. A similar behavior was seen between the trafficking of BODIPY–lac-Cer and another sphingolipid-interacting cargo, SBD (sphingolipid-binding domain) peptide, which when present together at the membrane trafficked nearly identically, but when incubated separately took very different routes (Steinert et al., 2008). An evaluation of the universality of hijacking by glycolipids, and its utility for cellular metabolism, must await further experiments.

**BODIPY–lac-Cer Trafficking in Drosophila Undergoes Pathway Switching in Response to Cholesterol Perturbations**

Importantly, this work has shown for the first time that in a fly neuronal culture system, the effects of cholesterol overloading on the trafficking and uptake of a simple glycolipid analogue, BODIPY–lac-Cer, is fundamentally similar to that in cholesterol-laden NPC fibroblasts, where lac-Cer trafficking is shifted in favor of the endolysosomal pathway. In contrast to human fibroblasts (Puri et al., 1999), however, fly neurons can still internalize BODIPY–lac-Cer after sterol depletion, making it possible to examine changes in trafficking that are associated with loss of cholesterol as well as cholesterol overload. An unexpected result of these experiments was that both cholesterol excess and sterol depletion resulted in aberrant trafficking of BODIPY–lac-Cer, with a strong accumulation in lysosomes under both conditions after several hours. The reasons for this are as yet unclear; however, it is not known whether otherwise normal human fibroblasts might react similarly to cholesterol depletion, because in the studies of Pagano and coworkers studies, fluorescent lac-Cer’s trafficking route could not be assayed because of a lack of uptake under these conditions.

In spite of the superficial similarity between the lysosomal accumulation of BODIPY–lac-Cer under cholesterol overload and depletion, it must be said that the trafficking route is clearly different when cholesterol levels are lowered, versus when they are raised (see Figure 7). Cholesterol excess allowed much more traffic through recycling compartments, but completely eliminated it from the Golgi. This is in contrast to sterol depletion, which causes complete bypass of recycling compartments, directly to sorting, Golgi, and lysosomal vesicles (summarized in Figure 7).
The results of the cholesterol overload and depletion experiments fit with a model where the uptake of glycolipid and subsequent choice between endolysosomal and recycling pathway is modulated by the level of sterol in these membranes and/or at the plasma membrane. Normally, most of the BODIPY–lac-Cer applied to the cells traffics quickly after sorting to recycling endosomes (see Figures 1 and 3), with the remainder being roughly split between late endolysosomes and Golgi. Cholesterol excess results in more BODIPY–lac-Cer being diverted gradually from a recycling pathway to accumulate in lysosomal compartments, leaving very little in the Golgi. This may be similar to the mammalian cholesterol storage situation, e.g., in NPC fibroblasts (Marks and Pagano, 2002). Under sterol depletion, the pathway is different: BODIPY–lac-Cer completely bypasses the recycling compartment and is shunted through sorting vesicles and then transiently through Golgi and finally accumulates in lysosomes. Changes in the targeting of BODIPY–lac-Cer under cholesterol perturbations may reflect a response of the cells to perceived nutritional state, causing a rerouting of internalized glycolipids that may accordingly alter lipid metabolism. As yet, a direct comparison between insect and mammalian cells’ response to cholesterol loss cannot be made; thus, we do not know whether the observations in the fly model under these conditions are applicable to mammalian cells. However, the similarities in the response of glycolipid trafficking to cholesterol excess, as is seen in the case of lipid storage diseases, strongly support Drosophila as a valid genetic system in which to model these diseases.

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REFERENCES

Baker, K. D., and Thummel, C. S. (2007). Diabetic larvae and obese flies: emerging studies of metabolism in Drosophila. Cell Metab. 6, 257–266.
Bard, F., et al. (2006). Functional genomics reveals genes involved in protein secretion and Golgi organization. Nature 439, 604–607.
Broeck, D. V., Lagrou, A. R., and De Wolf, M. J. (2007). Distinct role of clathrin-mediated endocytosis in the functional uptake of cholera toxin. Acta Biochim. Pol. 54, 757–767.
Choudhury, A., Dominguez, M., Puri, V., Sharma, D. K., Narita, K., Wheatley, C. L., Marks, D. L., and Pagano, R. E. (2002). Rab proteins mediate Golgi transport of caveola-internalized glycosphingolipids and correct lipid trafficking in Niemann-Pick C cells. J. Clin. Invest. 109, 1541–1550.
Choudhury, A., Sharma, D. K., Marks, D. L., and Pagano, R. E. (2004). Elevated endosomal cholesterol levels in Niemann-Pick cells inhibit rab4 and perturb membrane recycling. Mol. Biol. Cell 15, 4500–4511.
Costes, S. V., Delemans, D., Cho, E. H., Dobbim, Z., Pavlakis, G., and Lockett, S. (2004). Automatic and quantitative measurement of protein-protein colocalization in live cells. Biophys. J. 86, 3995–4003.
Cutler, R. G., Kelly, J., Storie, K., Pedersen, W. A., Tammarra, A., Hatanpaa, K., Troncoco, J. C., and Mattson, M. P. (2004). Involvement of oxidative stress-induced abnormalities in cereboid and cholesterol metabolism in brain aging and Alzheimer’s disease. Prog. Natl. Acad. Sci. USA 101, 2070–2075.
Deindhardt, K., Beminghausen, O., Willison, H. J., Hopkins, C. R., and Schiavo, G. (2006). Tetanus toxin is internalized by a sequential clathrin-dependent mechanism initiated within lipid microdomains and independent of epsin1. J. Cell Biol. 174, 459–471.
Finley, K. D., Edeeen, P. T., Cumming, R. C., Mandahl-Dumesnel, M. D., Taylor, B. J., Rodriguez, M. H., Hwang, C. E., Benedetti, M., and McKeown, M. (2003). blue cheese mutations define a novel, conserved gene involved in progressive neural degeneration. J. Neurosci. 23, 1254–1264.
Fluegel, M. L., Parker, T. J., and Pallanck, L. J. (2006). Mutations of a Drosophila NPC1 gene confer sterol and ecdysone metabolic defects. Genetics 172, 185–196.
Hebbar, S., Lee, E., Mannu, M., Steinert, S., Kumar, G. S., Wenk, M., Wohland, T., and Kraut, R. (2008). A fluorescent sphingolipid binding domain peptide probe interacts with sphingolipids and cholesterol-dependent raft domains. J. Lipid Res. 49, 1077–1089.
Holthuis, J.C.M., Pomorski, T., Raggers, R. J., Spong, H., and Van Meer, G. (2001). The organizing potential of sphingolipids in intracellular membrane transport. Physiol. Rev. 81, 1689–1723.
Huang, X., Suyama, K., Buchanan, J., Zhu, A. L., and Scott, M. P. (2005). A Drosophila model of the Niemann-Pick type C lysosome storage disease: dspcla is required for molting and sterol homeostasis. Development 132, 5115–5124.
Huang, X., Warren, J. T., Buchanan, J., Gilbert, L. I., and Scott, M. P. (2007). Drosophila Niemann-Pick type C 2 genes control sterol homeostasis and sterol biosynthesis: a model of human neurodegenerative disease. Development 134, 3733–3742.
Hung, W., and Geuze, H. J. (1996). Intracellular trafficking of lysosomal membrane proteins. Bioessays 18, 379–389.
Klein, U., Gimpil, G., and Fahrenholz, F. (1995). Alteration of the myometrial plasma membrane cholesterol content with beta-cyclodextrin modulates the binding affinity of the oxytocin receptor. Biochemistry 34, 13784–13793.
Koval, M., and Pagano, R. E. (1989). Lipid recycling between the plasma membrane and intracellular compartments: transport and metabolism of fluorescent sphingomyelin analogues in cultured fibroblasts. J. Cell Biol. 108, 2169–2181.
Lim, A., and Kraut, R. (2009). The Drosophila BEACH family protein, blue cheese, links lysosomal axon transport with motor neuron degeneration. J. Neurosci. 29, 951–963.
Macia, E., Ehrlich, M., Massol, R., Boucrot, E., Brunner, C., and Kirchhausen, T. (2006). Dynasore, a cell-permeable inhibitor of dynamin. Dev. Cell 10, 839–850.
Manders, E.M.M., Verbeek, F. J., and Aten, J. A. (1993). Measurement of co-localization of objects in dual-color confocal images. J. Microsc. 169, 375–382.
Marheineke, K., Grünewald, S., Christlieb, W., and Relländer, H. (1998). Lipid composition of Spodoptera frugiperda (SE9) and Trichoplusia ni (TN) insect cells used for baculovirus infection. FEBS Lett. 441, 49–52.
Marks, D. L., and Pagano, R. E. (2002). Endocytosis and sorting of glycosphingolipids in sphingolipid storage disease. Trends Cell Biol. 12, 603–613.
Martin, O. C., and Pagano, R. E. (1994). Internalization and sorting of a fluorescent analogue of glucosylceramides to the Golgi apparatus of human fibroblasts: utilization of endocytic and nonendocytic transport mechanisms. J. Cell Biol. 125, 769–781.
Massol, R. H., Larsen, J. E., Fujinaga, Y., Lencer, W. I., and Kirchhausen, T. (2004). Cholera toxin toxicity does not require functional Arf6- and dynamin-dependent endocytic pathways. Mol. Biol. Cell 15, 3631–3641.
Maxfield, F. R., and Tabas, I. (2005). Role of cholesterol and lipid organization in disease. Nature 438, 612–622.
Min, K. T., and Benzer, S. (1997). Spongecake and eggroll: two hereditary diseases in Drosophila resemble patterns of human brain degeneration. Curr. Biol. 7, 885–888.
Mukherjee, S., and Maxfield, F. R. (2004). Lipid and cholesterol trafficking in NPC. Biochim. Biophys. Acta 1685, 28–37.
Mylläryngas, L., Tyyynela, J., Page-McCaw, A., Rubin, G. M., Halita, M. J., and Feany, M. B. (2005). Cathepsin D-deficient Drosophila recapitulate the key features of neuronal ceroid lipofuscinoses. Neurobiol. Dis. 19, 194–199.
Nakano, Y., et al. (2001). Mutations in the novel membrane protein spinster interfere with programmed cell death and cause neural degeneration in Drosophila melanogaster. Mol. Cell. Biol. 21, 3775–3788.
Pagano, R. E. (2003). Endocytic trafficking of glycosphingolipids in sphingolipid storage diseases. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 358, 885–891.
Phillips, S. E., Woodruff, E. A., 3rd, Liang, P., Pattan, M., and Broadie, K. (2008). Neuronal loss of Drosophila NPC1a causes cholesterol aggregation and age-progressive neurodegeneration. J. Neurosci. 28, 6569–6582.

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Puglielli, L., Tanzi, R. E., and Kovacs, D. M. (2003). Alzheimer’s disease: the cholesterol connection. Nat. Neurosci. 6, 345–351.

Pulipparacharuvil, S., Akbar, M. A., Ray, S., Sevrioukov, E. A., Haberman, A. S., Rohrer, J., and Kramer, H. (2005). Drosophila Vps16A is required for trafficking to lysosomes and biogenesis of pigment granules. J. Cell Sci. 118, 3663–3673.

Puri, V., Jefferson, J. R., Singh, R. D., Wheatley, C. L., Marks, D. L., and Pagano, R. E. (2003). Sphingolipid storage induces accumulation of intracellular cholesterol by stimulating SREBP-1 cleavage. J. Biol. Chem. 278, 20961–20970.

Puri, V., Watanabe, R., Dominguez, M., Sun, X., Wheatley, C. L., Marks, D. L., and Pagano, R. E. (1999). Cholesterol modulates membrane traffic along the endocytic pathway in sphingolipid-storage diseases. Nat. Cell Biol 1, 386–388.

Puri, V., Watanabe, R., Singh, R. D., Dominguez, M., Brown, J. C., Wheatley, C. L., Marks, D. L., and Pagano, R. E. (2001). Clathrin-dependent and -independent internalization of plasma membrane sphingolipids initiates two Golgi targeting pathways. J. Cell Biol. 154, 535–547.

Rietveld, A., Neutz, S., Simons, K., and Eaton, S. (1999). Association of sterol- and glycosylphosphatidylinositol-linked proteins with Drosophila raft lipid microdomains. J. Biol. Chem. 274, 12049–12054.

Sabharanjak, S., Sharma, P., Parton, R. G., and Mayor, S. (2002). GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. Dev. Cell 2, 411–423.

Seppo, A., Moreland, M., Schweingruber, H., and Tiemeyer, M. (2000). Zwitterionic and acidic glycosphingolipids of the Drosophila melanogaster embryo. Eur. J. Biochem. 267, 3549–3558.

Sharma, D. K., Brown, J. C., Choudhury, A., Peterson, T. E., Holicky, E., Marks, D. L., Simari, R., Parton, R. G., and Pagano, R. E. (2004). Selective stimulation of caveolar endocytosis by glycosphingolipids and cholesterol. Mol. Biol. Cell 15, 3114–3122.

Sharma, D. K., Choudhury, A., Singh, R. D., Wheatley, C. L., Marks, D. L., and Pagano, R. E. (2003). Glycosphingolipids internalized via caveolar-related endocytosis rapidly merge with the clathrin pathway in early endosomes and form microdomains for recycling. J. Biol. Chem. 278, 7564–7572.

Silence, D. J., Puri, V., Marks, D. L., Butters, T. D., Dwek, R. A., Pagano, R. E., and Platt, F. M. (2002). Glucosylceramide modulates membrane traffic along the endocytic pathway. J. Lipid Res. 43, 1837–1845.

Simonsen, A., Cumming, R. C., Lindmo, K., Galaviz, V., Cheng, S., Rusten, T. E., and Finley, K. D. (2007). Genetic modifiers of the Drosophila blue cheese gene link defects in lysosomal transport with decreased life span and altered ubiquitinated-protein profiles. Genetics 176, 1283–1297.

Singh, R. D., Puri, V., Valiayaveettil, J. T., Marks, D. L., Bittman, R., and Pagano, R. E. (2003). Selective caveolin-1-dependent endocytosis of glycosphingolipids. Mol. Biol. Cell 14, 3254–3265.

Sriram, V., Krishnan, K. S., and Mayor, S. (2003). Deep-orange and carnation define distinct stages in late endosomal biogenesis in Drosophila melanogaster. J. Cell Biol. 161, 593–607.

Steinert, S., et al. (2008). A fluorescent glycolipid-binding peptide probe traces cholesterol dependent microdomain-derived trafficking pathways. PLoS ONE 3, e2933.

Sillence, D. J., Puri, V., Marks, D. L., Butters, T. D., Dwek, R. A., Pagano, R. E., and Platt, F. M. (2002). Glucosylceramide modulates membrane traffic along the endocytic pathway. J. Lipid Res. 43, 1837–1845.

Simonsen, A., Cumming, R. C., Lindmo, K., Galaviz, V., Cheng, S., Rusten, T. E., and Finley, K. D. (2007). Genetic modifiers of the Drosophila blue cheese gene link defects in lysosomal transport with decreased life span and altered ubiquitinated-protein profiles. Genetics 176, 1283–1297.

Singh, R. D., Puri, V., Valiayaveettil, J. T., Marks, D. L., Bittman, R., and Pagano, R. E. (2003). Selective caveolin-1-dependent endocytosis of glyco- sphingolipids. Mol. Biol. Cell 14, 3254–3265.

Sriram, V., Krishnan, K. S., and Mayor, S. (2003). deep-orange and carnation define distinct stages in late endosomal biogenesis in Drosophila melanogaster. J. Cell Biol. 161, 593–607.

Steinert, S., et al. (2008). A fluorescent glycolipid-binding peptide probe traces cholesterol dependent microdomain-derived trafficking pathways. PLoS ONE 3, e2933.

te Vruchte, D., Lloyd-Evans, E., Veldman, R. J., Neville, D. C., Dwek, R. A., Platt, F. M., van Blitterswijk, W. J., and Silence, D. J. (2004). Accumulation of glycosphingolipids in Niemann-Pick C disease disrupts endosomal transport. J. Biol. Chem. 279, 26167–26175.

Tschape, J. A., Hammerschmied, C., Muhlig-Versen, M., Athenstaedt, K., Daum, G., and Kretzschmar, D. (2002). The neurodegeneration mutant lochrig interferes with cholesterol homeostasis and Apol processing. EMBO J. 21, 6367–6376.

Ui, K., Nishihara, S., Sakuma, M., Togashi, S., Ueda, R., Miyata, Y., and Miyake, T. (1994). Newly established cell lines from Drosophila larval CNS express neural specific characteristics. In Vitro Cell Dev. Biol. Anim. 30A, 209–216.

Wiegandt, H. (1992). Insect glycolipids. Biochim. Biophys. Acta Lipids Lipid Metab. 1123, 117–126.

Wolozin, B. (2004). Cholesterol and the biology of Alzheimer’s disease. Neuron 41, 7–10.

Zhai, L., Chaturvedi, D., and Cumberledge, S. (2004). Drosophila wnt-1 undergoes a hydrophobic modification and is targeted to lipid rafts, a process that requires porcupine. J. Biol. Chem. 279, 33220–33227.