

GLYCOSPHINGOLIPID-AIDED INTRACELLULAR DELIVERY OF CARGO MOLECULES

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims benefit of, and priority from, U.S. provisional patent application No. 60/905,805, filed on March 9, 2007, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The present invention relates generally to methods of delivering cargo molecules into live cells.

BACKGROUND OF THE INVENTION

[0003] The uptake and intracellular trafficking of sphingolipids, including glycosphingolipids, is associated with many pathological conditions, including viral infection, toxin poisoning, lipid storage disease, neurodegenerative disease, and inflammation.

[0004] Sphingolipid and cholesterol trafficking is altered in the cells of patients with Niemann Pick disease, and a number of other lipid storage diseases where sphingolipids accumulate in late endosomal and lysosomal compartments (Pagano, 2003; Simons, 2000). Cholesterol and sphingolipids such as ceramide, sphingomyelin, and gangliosides are also thought to be involved in the pathogenesis of Alzheimer's disease (Cutler, 2004; Han, 2005; Mattson, 2005; Soreghan, 2003).

[0005] In particular, sphingolipid trafficking in cells is strongly perturbed by endolysosomal lipid storage that occurs in neurodegenerative lipid storage and metabolism diseases and disorders (Marks and Pagano, 2002). Such diseases and disorders include Niemann-Pick Type C (NPC), Alzheimer's disease, neuronal ceroid lipofuscinosis, Batten's disease, GM2 gangliosidosis including AB variant, Sandhoff disease, Tay-Sachs disease, GM1 gangliosidosis, mucopolipidosis type IV, Gaucher's disease, Farber disease, Fabry's disease, metachromatic leukodystrophy, and Krabbe disease.

[0006] Glycosphingolipid uptake and trafficking within a cell have been shown to

be affected by lipid perturbations (for example, cholesterol storage). 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 evaluate the effectiveness of treatments.

[0007] Fluorescently labeled glycosphingolipids are potentially valuable tools in the characterization of lipid storage disorders (Huang et al., 2005; Min and Benzer, 1997; Myllykangas et al., 2005; Nakano et al., 2001; Sweeney and Davis, 2002), and other diseases that affect lipid storage and metabolism, such as Alzheimer's disease (Cutler et al., 2004; Maxfield and Tabas, 2005; Puglielli et al., 2003; Wolozin, 2004).

[0008] Further tools and methods to diagnose, monitor and treat lipid storage and neurodegenerative diseases are desirable.

SUMMARY OF THE INVENTION

[0009] The present methods relate to exploitation of a newly discovered phenomenon in the trafficking behavior of glycosphingolipids. The inventors have discovered that glycosphingolipids divert the trafficking pathway of an endocytic cargo completely away from its normal lysosomal target. The finding that when glycosphingolipids interact with a cargo, both are directed to an alternate intracellular path, provides the foundation for new methods that may be useful to target drugs and therapeutic agents for delivery to a cellular location other than the degradative lysosome.

[0010] The present methods may be performed using a fluorescently tagged glycosphingolipid which, due to the single fluorescence emission wavelength of the attached fluorophore, allows quantitative and reliable colocalization with a second fluorescent label in the same cell.

[0011] In one aspect of the present invention, there is provided a method of delivering an endocytic cargo to a cellular compartment comprising contacting a glycosphingolipid and the endocytic cargo with the external surface of a cell so that the glycosphingolipid and the endocytic cargo are taken up into the cell and targeted

to a non-lysosomal compartment.

[0012] The glycosphingolipid may comprise lactosyl ceramide. The cell may be in vitro, including a cell in culture or an explanted cell from a subject, or may be in vivo.

[0013] The glycosphingolipid or the endocytic cargo or both may comprise a detectable label, including a detectable label comprising a fluorescent group. In particular, the detectable label may comprise BODIPY-Far Red 650/665.

[0014] Thus, the method may further comprise detecting a label on the glycosphingolipid, the endocytic cargo, or both. Where one or both labels comprise a fluorescent group, detecting may be performed by visualizing either fluorescent group using fluorescence microscopy.

[0015] In particular, the cell may be a cell from a subject for diagnosis of a sphingolipid related disorder. The method may then further comprise comparing the glycosphingolipid trafficking pattern in the cell with the glycosphingolipid trafficking pattern observed for a healthy cell.

[0016] The endocytic cargo may comprise a therapeutic agent, including a drug, an antibiotic, a hormone, a cellular factor, a nucleic acid molecule or a polypeptide.

[0017] Thus, the method may entail contacting an effective amount of the endocytic cargo comprising the therapeutic agent with the external surface of the cell of a subject in need of treatment of a sphingolipid related disorder or a disorder in which the endocytic cargo comprising a therapeutic agent is desired to be re-directed within a cell.

[0018] The sphingolipid related disorder to be diagnosed may be a neurodegenerative disorder, including Alzheimer's disease.

[0019] Other aspects and features of the present invention will become apparent to those of ordinary skill in the art upon review of the following description of specific embodiments of the invention in conjunction with the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] In the figures, which illustrate, by way of example only, embodiments of the present invention,

[0021] **Figure 1: BODIPY-labelled LacCer analogs are taken up into endocytic vesicles in *Drosophila* neurons** (A) Lac-Cer is incorporated into the plasma membrane and endocytosed in *Drosophila* neurons incubated with a mixture of FR-LacCer and FL-LacCer. The attached fluorophore (BODIPY 650-665 FR vs BODIPY 505-511 FL) does not influence the uptake or subcellular trafficking of lac-Cer since the two labels traffic identically as can be seen by nearly complete overlap of the FL (green) and FR (blue) signals. Lac-Cer carrying compartments are highly mobile, typical for endosomes. Scalebar = 8 μm . (B) Transfected LAMP-GFP (green) and lac-Cer-FR (blue) colocalization. Scalebar = 5 μm . (C) Transfected LAMP-GFP and pulsed FR-Lac-Cer colocalization shows a peak in presumptive sorting endosomes at ~30 min after uptake, falling to ~15% at the sorting to late endosome transition (60-90min). (D) A non-glycosylated sphingolipid TR-Ceramide (red) also colocalizes to a limited extent with the lysosomal markers Dextran and LysoTracker (green; not quantified).

[0022] **Figure 2: LacCer traffics primarily through recycling endosomes, and is only partially Golgi associated** (A, B) FR-lac-Cer (blue) colocalization with transfected MannII-GFP (green). After 60min, Lac-Cer traffics only partially (~15-20%) to cis and medial Golgi compartments, labeled by MannII-GFP. Bars = 5 μm . (C,D) FR-lac-Cer (blue) colocalization with rab11-GFP (green)-positive recycling endosomes. Roughly twice the amount of lac-Cer (35-40%) traffics to recycling endosomes as to Golgi. Scalebars = 4 μm .

[0023] **Figure 3: LacCer and Dextran traffic identically when present together** (A, B) Simultaneous labelling of FL-lac-Cer with Alexa647 (FR)-Dextran resulted in very high colocalization. In contrast, sequential labelling of FL-lac-Cer and 3 h later with Dextran results in essentially no co-localization between the two dyes, even given sufficient chasing time that a substantial portion of each label by itself would have reached late endosomes/lysosomes. Bars = 8 μm . (C) Co-localization values are continuously high over time after simultaneous incubation

of lac-Cer and Dextran (black trace), compared with that seen between Dextran and another endolysosomal marker, LAMP-GFP (red trace; see Figure 1); “simultaneous” refers to addition of Dextran directly after washing of the FL-lac-Cer. (D) The order of sequential incubation has no effect on the co-localization values. Co-localization is significantly lower when sequentially incubated, indicating that lac-Cer is taken up via a different endocytic pathway from Dextran. Asterisk (*) denotes $P < 0.05$ obtained from Student’s t-test by comparing the datasets from simultaneous versus sequential incubation. (E) Lac-Cer influences trafficking of Dextran independent of the relative concentrations of both dyes, indicating that the altered transport is not due to direct binding. Cells were incubated with 5 μ M FL-LacCer and 0.5 mg/mL Alexa647-Dextran (“5:5”), 5 μ M LacCer and 0.1 mg/mL Dextran (“5:1”) and 1 μ M LacCer and 0.5 mg/mL Dextran (“1:5”) simultaneously and co-localization values were calculated after 180 min.

[0024] Figure 4: LacCer uptake in the presence of Dextran alters trafficking itineraries of both tracers Endolysosomes, Golgi, and lysosomes were labeled, respectively, with LAMP-GFP (A), MannII-GFP (B), and Alexa546-Dextran chased for 15h (C), and subsequently labeled with FL-LacCer and Alexa647-Dextran simultaneously to determine whether either label changes its trafficking route in the presence of the other. (A) Both labels accumulate slowly in LAMP-positive compartments, more than would be expected for lac-Cer, but less than Dextran by itself. (B) Trafficking of FR-Dextran and lac-Cer to lysosomes labeled by 15h incubation of red(Alexa@546)-Dextran was completely inhibited in the presence of lac-Cer (black trace); compare to strong colocalization of Dextran in the absence of lac-Cer (~5% vs. 80% after 4h). (C) Trafficking of lac-Cer to Golgi (normally ~20%; red trace) is also inhibited by the presence of Dextran (black trace). Dextran does not normally traverse the Golgi, as indicated by (blue) trace in the absence of lac-Cer.

[0025] Figure 5: Lac Cer trafficking to Golgi vs. EL pathway is controlled by a cholesterol switch (A, B) Cholesterol depletion leads to a transient enhancement of lac-Cer accumulation in Golgi (labeled by MannII-GFP), peaking at 180min. The subsequent drop in Golgi colocalization at 240min could result from catabolism of lac-Cer to other labeled products. Bars = 4 μ m. (C) Trafficking to endolysosomal compartments labeled by LAMP-GFP is also increased under cholesterol depletion.

Cholesterol depletion appears to suppress efficiency of uptake somewhat in both A and B (15-60min), but the differences from controls are not significant. (D) Lac-Cer (blue) accumulates aberrantly in enlarged LAMP-positive (green) compartments under cholesterol excess (red trace). Bars = 4 μ m. (E) Cholesterol excess leads to the converse effect of that from cholesterol depletion on trafficking of lac-Cer to Golgi, which is nearly completely suppressed (contrast with B). (F) Trafficking of lac-Cer to endolysosomal compartments labeled by LAMP-GFP is strongly increased under cholesterol excess, similar to the effect of cholesterol depletion.

[0026] **Scheme 1:** Schematic diagram of steps involved in synthesis of BODIPY650/665-lac-Cer (Far-red BODIPY-lac-Cer or FR-BODIPY-lac-Cer).

DETAILED DESCRIPTION

[0027] The presently described methods relate to the novel approach of adding a glycosphingolipid to the surface of a cell in the presence of an endocytic cargo molecule, which has been found to lead to altered trafficking of the endocytic cargo once taken up by the cell. This phenomenon is exploited as the basis for methods for directing the uptake of therapeutic drugs and agents to a non-degradative cellular location. As well, monitoring the uptake and trafficking of a single-emission fluorescently labelled glycosphingolipid, for example BODIPY-FR-lactosyl ceramide, provides the basis for methods for monitoring trafficking defects including those arising in diseases related to lipid storage and metabolism, including neurodegenerative disorders.

[0028] Briefly, exogenous glycosphingolipids that are externally applied to the surface of a cell are taken up by the cell and traffic internally within the cell to various cellular compartments. For example, lac-Cer is taken up by the cells and is then trafficked to endosomes and to the Golgi. Similarly, an exogenous endocytic tracer, such as Dextran, is taken up by cells and trafficked through endocytic compartments, but it reaches a different cellular compartment from lac-Cer, namely the lysosome, which is a degradative organelle. As is now described, simultaneous application of the glycosphingolipid with a cargo to the cell surface results in redirection of the cargo. An endocytic cargo is typically directed via an endosomal trafficking pathway to lysosomes when applied to the cell in the absence of the glycosphingolipid,

meaning that the cargo is targeted for degradation. Surprisingly, as demonstrated by the inventors of the present methods, simultaneous application of the glycosphingolipid re-directs at least a portion of the endocytic cargo away from the endocytic lysosomes to a non-degradative compartment.

[0029] The ability to steer the intracellular trafficking pathway of an endocytic cargo molecule that is added simultaneously with a glycosphingolipid to cells is useful for applications that target agents to a non-lysosomal cellular location, for example the targeting of drugs or therapeutic agents in drug delivery applications. Many cargoes are substantially degraded in lysosomes after uptake, reducing their potential efficacy within the cell. Thus, these methods may be used to steer a cargo away from lysosomal degradative pathways and toward cellular locations where degradation does not take place, such as the Golgi or sorting endosomes, thus potentially improving the efficacy of an agent delivered to a cell for diagnostic or therapeutic purposes.

[0030] This phenomenon of the addition of an exogenous glycosphingolipid having a steering effect on the intracellular trafficking of an endocytic cargo has not previously been described.

[0031] It has previously been recognized that addition of exogenous ceramides, including lactosyl-ceramide and other short-chain ceramides, stimulates the caveolar endocytic pathway. Pagano et al. devised the first fluorescently tagged sphingolipid and glycosphingolipid analogs, including lactosyl ceramide (Martin and Pagano, 1994). These authors documented the fact that these lipid analogs were trafficked normally in cells, and in the case of the fluorescently labeled lactosyl ceramides, metabolized like endogenous lipids. They went on to characterize alterations in the intracellular trafficking of lactosyl ceramide under cellular conditions brought about by lipid and cholesterol storage diseases, in human lipid storage diseased fibroblasts. It was found that lactosyl-ceramide underwent a drastic change in its trafficking behaviour in the mutant cells, where it accumulated in degradative (endolysosomal) as opposed to biosynthetic (Golgi) compartments. This effect was reversible by reductions in cholesterol.

[0032] Accordingly, there is presently provided a method of delivering an

endocytic cargo molecule to a cellular compartment, using an externally applied glycosphingolipid to direct the trafficking of the cargo molecule within the cell.

[0033] The method comprises the addition, either simultaneously or in rapid succession, including in either order, of a glycosphingolipid and an endocytic cargo to a cell so that the glycosphingolipid and the endocytic cargo are both taken up into the cell.

[0034] A glycosphingolipid refers to a lipid having a shingosine amino alcohol group and a sugar head group. The glycosphingolipid used may be any glycosphingolipid that is taken up by a cell when applied externally to the cell. For example, the glycosphingolipid may be a cerebroside, a ganglioside, a globoside, sphingomyelin, myelin, lactosyl ceramide (lac-Cer), glucosyl ceramide, galactosyl ceramide, GD2, GD1a, GD1b, GM1 or a combination of two or more of these glycosphingolipids. In a particular embodiment, the glycosphingolipid is a glycosylated ceramide, particularly glycosyl ceramide, galatosyl ceramide, and particularly lac-Cer.

[0035] In order to monitor or track the path of the externally applied glycosphingolipid after it is taken up by the cell and is trafficked within the cell through various cellular compartments, the glycosphingolipid may be coupled to a detectable label, for example a small molecule fluorescent tag, which allows for detection of the glycosphingolipid once taken up into living cells, in a non-invasive manner.

[0036] By labeling the glycosphingolipid, a convenient method is provided for monitoring the intracellular trafficking of the glycosphingolipid as it is transported between compartments in living cells. This allows for monitoring of changes in the glycosphingolipid trafficking that may occur in cellular models of disease states, including neurodegenerative diseases and other diseases linked with metabolism, storage and trafficking of lipids. Labeling of the glycosphingolipid also allows for detection of the localization of the glycosphingolipid in the cell, assisting in determination of the position of the glycosphingolipid compared to the position of an endocytic cargo that is to be re-directed in the cell.

[0037] Detectable label refers to any tag or label that can be detected by any

means, directly or indirectly, for example by using visualizing methods, autoradiography methods, colour development methods or by affinity binding. It will be appreciated that the detectable label selected should not interfere with the ability of the glycosphingolipid to be taken up by a cell upon external application to the cell surface or the trafficking of the glycosphingolipid within the cell once taken up. For example, the tag or label may comprise a fluorescent group, a chemiluminescent group, a radioactive group, a ligand (for example biotin), a photolabile fluorescent group, a paramagnetic group, or a heavy metal complex or moiety.

[0038] In a particular embodiment, the detectable label is a fluorescent group, such as a fluorophore. This allows for direct visualization of the probe when associated with a sphingolipid using fluorescence microscopy techniques, including when associated with a sphingolipid contained within a cell. Inclusion of a single emission fluorophore allows for use of the probe in double visualization experiments, for example in which a second fluorophore is used to target a different class of molecules or a specific organellar compartment in the cell.

[0039] Any fluorophore may be chosen, for example BODIPY dyes, Alexa dyes, TAMRA, Oregon Green or a quantum dot. In particular, the fluorescent group may particularly be a far red-emitting fluorophore, in a particular example BODIPY-FR 650/665® (Invitrogen).

[0040] Most existing markers used to detect various cellular compartments are available as fluorescent fusion proteins, or emit red fluorescence, which overlap with one of the emission wavelengths of commonly used fluorophore BODIPY FL, making determination of the localization of the glycosphingolipid once located within the cell potentially ambiguous. A far-red emitting fluorescent label such as BODIPY-FR 650/665® fluoresces in the far red range (650-665 nm) only, which does not overlap with green emissions of standard green fluorophores or green fluorescent protein (GFP). This allows for localization of the glycosphingolipid in relation to cellular markers labelled with these other common (usually green-emitting) fluorophores, allowing for multiple labeling of a cell with other fluorophores that would otherwise not give separable fluorescence emission signals.

[0041] In particular, BODIPY-FR labeled glycosphingolipid, including BODIPY-

FR-lac-Cer, serves as a marker molecule to enable monitoring of trafficking patterns in healthy and diseased cells, enabling the diagnosis, detection, tracking of the progression of and the effect of treatment of disease states such as disorders or diseases involving defects in lipid storage, lipid trafficking or lipid metabolism, including neurodegenerative disorders.

[0042] The endocytic cargo molecule may be any molecule that, when externally applied to the surface of a cell, is taken up into endocytic vesicles, and that is desired to be delivered to a cellular compartment, for example a subset of vesicles, or a particular organelle other than the lysosome.

[0043] Alternatively, the endocytic cargo may comprise or consist of a therapeutic agent that is to be delivered into a cell, for which the trafficking path of the endocytic cargo when taken up into a cell in the absence of the exogenous glycosphingolipid is desired to be altered or re-directed using the influence of the glycosphingolipid. For example, the endocytic cargo may be any agent having a therapeutic or preventative effect or which effects a desired therapeutic result, that is to be delivered into a cell via the endocytic pathway. For example, the therapeutic agent may comprise a drug, an antibiotic, a hormone, a cellular factor, a nucleic acid molecule or a polypeptide.

[0044] In order to track the progression of the endocytic cargo, the endocytic cargo molecule may be labeled with a detectable label, as described above for the glycosphingolipid. The detectable label for the endocytic cargo may be detectable by the same or a different technique than that used to detect a detectable marker on the sphingolipid. If the endocytic marker and glycosphingolipid are to be detected together or using the same techniques, it will be appreciated that the respective detectable labels should be chosen to allow the two to be distinguished from each other and from any other labeled cellular markers that may be used in the method.

[0045] The glycosphingolipid and the endocytic cargo are contacted with the external surface of a cell into which the two are to be taken up via endocytic mechanisms, for example by addition of the glycosphingolipid and the endocytic cargo to a solution or medium containing the cell, or by addition to a cell culture.

[0046] The glycosphingolipid and the endocytic cargo are contacted with the external surface of the cell at the same time, or for overlapping periods of time during

which the cell surface is contacted with each of the glycosphingolipid and the endocytic cargo, or for periods of time directly following one another during which the cell surface is contacted with each of the glycosphingolipid and the endocytic cargo, including for allowance of a brief wash of the cell surface between the two periods of contact. As stated above, if the glycosphingolipid and the endocytic cargo are added one after another or for overlapping periods of time, the glycosphingolipid and the endocytic cargo may be added in either order.

[0047] The cell surface is contacted with the glycosphingolipid and the endocytic cargo for a period of time sufficient to allow the glycosphingolipid and the endocytic cargo to adhere to the cell surface and to be ultimately taken up into endocytic vesicles. For example, the glycosphingolipid may be contacted with the cell for a period of time from about 1 minute to about an hour, or from about 5 minutes to about 45 minutes, or from about 15 minutes to about 30 minutes, or for about 30 minutes. The endocytic cargo may be contacted with the cell for a period of time from about 1 minute to about 30 minutes, or for about 2 minutes to about 15 minutes, or for about 5 minutes.

[0048] The glycosphingolipid may be added to a solution, medium or cell culture at a concentration of at least about 0.1 μM , at least about 1 μM , at least about 2.5 μM , at least about 5 μM , at least about 10 μM , at least about 25 μM , at least about 50 μM , at least about 75 μM , at least about 100 μM , from about 0.1 μM to about 100 μM , from about 1 μM to about 50 μM , from about 5 μM to about 25 μM , or from about 1 μM to about 10 μM .

[0049] The molar ratio of glycosphingolipid to endocytic cargo may be, for example, from about 20:1 to about 1:20, from about 10:1 to about 1:10, from about 7.5:1 to about 1:7.5, from about 5:1 to about 1:5, from about 4:1 to about 1:4, from about 3:1 to about 1:3, from about 2:1 to about 1:2, or about 1:1.

[0050] The cell may be an in vitro cell, including a cell in culture or an explanted cell from a subject, or may be an in vivo cell. In a particular embodiment, the cell is a neuron. In a particular embodiment, the cell is a cell to which an endocytic cargo is to be delivered, including an endocytic cargo comprising a therapeutic agent. In another particular embodiment, the cell is a cell associated with or that is known to cause a

particular disorder or disease that is being monitored, diagnosed or treated, including neurodegenerative diseases and other diseases linked with metabolism, storage and trafficking of lipids.

[0051] Reference to "a cell" is intended to include reference to a single cell, a plurality of cells or a population of cells, unless otherwise specified. Similarly, where applicable, reference to "cells" is intended to refer to a plurality of cells or a population of cells and also to apply to a single cell, where applicable. A healthy cell is a cell that is known not to have a disorder, mutation, or malfunction that is found in a cell associated with or that is known to cause a particular disorder or disease that is being monitored, diagnosed or treated, including neurodegenerative diseases and other diseases linked with metabolism, storage and trafficking of lipids.

[0052] If the method of delivery of an endocytic target is to be to monitor or track the path of the externally applied glycosphingolipid after it is taken up by the cell and is trafficked within the cell through various cellular compartments, the glycosphingolipid and/or the endocytic cargo may comprise a detectable label as described above. The method thus further includes the step of detecting the detectable label on the glycosphingolipid, and may also further comprise detecting the detectable label on the endocytic cargo.

[0053] The method of detecting each label will depend on the nature of the labels themselves. Methods for detecting various types detectable labels are known in the art, and include fluorescent, radiographic, immunoassay, enzyme-linked, or colourimetric methods, for example.

[0054] - If the detection is to be done in a cell, then each of the detectable labels may conveniently be a fluorescent label. The glycosphingolipid and/or the endocytic cargo, when including a fluorescent label, may each be directly visualized by fluorescence microscopy. As mentioned above, it will be appreciated that each label may be selected so that the labels can readily be distinguished from each other if the labels are to be detected by the same detection technique.

[0055] By detecting the fluorescent label over time, it is possible to track the path of the glycosphingolipid and/or endocytic cargo through the cell, and thus monitor glycosphingolipid and/or endocytic cargo trafficking from the cell surface plasma

membrane to various locations within the cell via endocytic vesicles as the endocytic vesicles are internalized and as the glycosphingolipid and/or endocytic cargo are then subsequently trafficked to various cellular compartments and locations.

[0056] The presently described methods provide a valuable tool for studies aimed at assessing changes in glycosphingolipid trafficking in cellular models of neurodegenerative disease and other disorders affecting lipid metabolism, lipid storage and lipid trafficking.

[0057] The present methods may be used in screens to recognize drug-induced recovery from abnormal localization of a fluorescently labeled lac-Cer in relation to a stably or transiently expressed fluorescent marker of the relevant compartment(s) in a given cell line. To this end, a detectable label such as a single-emission fluorophore (for example BODIPY-FR 650/665) on the glycosphingolipid provides ease of detection of the glycosphingolipid without interference or overlap with a fluorescent marker used to visualize a particular cellular compartment.

[0058] As described in the following examples, glycosphingolipid uptake and trafficking has been demonstrated to occur in a similar fashion in *Drosophila* and mammalian neurons, indicating that the present methods may be performed in *Drosophila* cells, which serve as a useful tool for studying lipid trafficking and storage disease models.

[0059] Such intracellular monitoring of glycosphingolipid trafficking may be particularly useful in diagnosis of disease states. For example, live observation of the trafficking of glycosphingolipids in afflicted neurons compared to healthy neurons would allow for detection of sphingolipid related disorders, such as neurodegenerative diseases.

[0060] Thus, the method of targeting may further include diagnosis of a sphingolipid related disorder, including a lipid storage or lipid trafficking related disorder.

[0061] Detecting of a detectable marker may be done in a cell from a subject to be diagnosed, including a cell explanted from the subject. Such detection may be combined with the methods as described herein, in which the glycosphingolipid and

the endocytic cargo are both labeled with a detectable label. The re-direction of the endocytic cargo can be monitored by detection of both the labeled glycosphingolipid and the labeled endocytic cargo. The trafficking pattern of glycosphingolipids and endocytic cargo in the subject's cell may be compared with the pattern observed for a healthy cell, where a disrupted or altered pattern in the subject's cell is indicative of a sphingolipid related disorder. For example, the rate, localization, or extent of trafficking can be compared.

[0062] A sphingolipid related disorder as used herein refers to any disease, disorder or condition which is associated with, related to, or a characteristic of which is anomalous or aberrant sphingolipid (including glycosphingolipid) storage, metabolism, trafficking, production, localization or composition, or which depends on sphingolipid recognition and uptake for its infectivity or etiology. For example, the disease state may be a neurodegenerative disorder, including Alzheimer's disease or a prion related disease, or a lipid storage disorder such as Niemann-Pick type C, neuronal ceroid lipofuscinosis, Batten's disease, GM2 gangliosidosis including AB variant, Sandhoff disease, Tay-Sachs disease, GM1 gangliosidosis, mucopolidosis type IV, Gaucher's disease, Farber disease, Fabry's disease, metachromatic leukodystrophy and Krabbe disease.

[0063] The method of delivering an endocytic cargo as described herein may also be a method of treating a disorder or disease, including a sphingolipid related disorder or any disorder or disease in which the endocytic cargo comprising a therapeutic agent is desired to be re-directed within a cell.

[0064] Thus, an effective amount of an endocytic cargo comprising a therapeutic agent is contacted with a cell of a subject in need of such treatment in accordance with the methods as described herein, including an explanted cell or cell in vivo.

[0065] "Treating" a disease state refers to an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent of disease, stabilization of the state of disease, prevention of development of disease, prevention of spread of disease, delay or slowing of disease progression, delay or slowing of disease onset, amelioration or

palliation of the disease state, and remission (whether partial or total). "Treating" can also mean prolonging survival of a patient beyond that expected in the absence of treatment. "Treating" can also mean inhibiting the progression of disease, slowing the progression of disease temporarily, although more preferably, it involves halting the progression of the disease permanently.

[0066] The term "effective amount" as used herein means an amount effective, at dosages and for periods of time necessary to achieve the desired result (e.g. to treat a sphingolipid related disorder, including a neurodegenerative disorder, including Alzheimer's disease).

[0067] The cell to which the endocytic cargo comprising a therapeutic agent is administered may be an explanted cell from a subject, or may be an in vivo cell. Where the cell is an explanted cell, the treatment may further comprise implanting the cell back into the subject following ex vivo contacting of the glycosphingolipid and endocytic cargo to the cell.

[0068] There is presently provided various uses of an endocytic cargo comprising a therapeutic agent, a detectable label, or both, including use of such an endocytic cargo to diagnose a sphingolipid related disorder, use of the endocytic cargo to treat a disorder where treatment of the disorder exploits the currently described phenomenon of re-directing an endocytic target within a cell by contacting the endocytic cargo in combination with a glycosphingolipid, and use of an endocytic cargo in the manufacture of a medicament for diagnosis or treatment of a disorder where treatment of the disorder exploits the currently described phenomenon of re-directing an endocytic target within a cell by contacting the endocytic cargo in combination with a glycosphingolipid.

[0069] The present methods and uses are further exemplified by way of the following non-limited examples.

EXAMPLES

[0070] EXAMPLE 1

[0071] Introduction

[0072] This study identifies a novel phenomenon whereby 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. It is proposed to call this phenomenon "hijacking" because it involves the re-routing of the trafficking of one agent by the other.

[0073] To address the question of how GSLs are trafficked in *Drosophila*, fluorescent analogs of a simple glycosphingolipid, lac-Cer, were used to show that a *Drosophila* neuronal cell line incorporates and traffics glycosphingolipids in a manner analogous to that observed in human cell lines. Time course pulse-chase experiments with fluorescent lac-Cer were carried out on cells transfected with or treated with endolysosomal, Golgi, and recycling endosome markers, and it was established that the lac-Cer remains predominantly in recycling endosomes. A smaller contribution is trafficked to the Golgi through presumptive sorting endosomes. Additionally, a minor proportion of fluorescently tagged ceramide traffics to late endosomal vesicles and lysosomes.

[0074] It was also established in this study that cholesterol manipulations of *Drosophila* neuronal cells, both overload and depletion, influence the trafficking behavior of lac-Cer. With respect to cholesterol overload, this effect is analogous to that which has been reported in the case of NPC fibroblasts by Pagano et al, i.e. that lac-Cer accumulates aberrantly in an endolysosomal compartment (Pagano, 2003). Surprisingly, it was found that cholesterol depletion causes a similar accumulation of lac-Cer in endolysosomes, but also transiently increases it in Golgi.

[0075] Thus, this study demonstrates that glycosphingolipid trafficking in *Drosophila* neurons follows similar routes to that described in mammalian cells, and importantly, that its behaviors are strongly influenced by conditions of cholesterol storage.

[0076] Materials and Methods

[0077] Cell Culture: Neuronal *Drosophila melanogaster* c6 cells (DL-DmBG2-c6) were obtained from the *Drosophila* Genome Resource Center (DGRC; Indiana, USA) and cultured at 25°C in Schneider's medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA), 0.125 IU/ml Insulin (Biological Industries, Israel) and 1%

antibiotic/antimycotic solution (Gibco, USA):

[0078] Synthesis of BODIPY-FR 650/665-lac-Cer: Far-red BODIPY-FR 650/665-lac-Cer was synthesized as follows (see scheme 1): A flame dried 5 mL reaction vial was charged with *D*-lactosyl-*beta*-1'-*D*-erythro-Sphingosine (4.87 mg, 0.0078 mmol), BODIPY 650/665-X (5 mg, 0.0078 mmol) under argon atmosphere and was cooled in an ice bath at 0 °C. Dry dimethyl formamide (DMF, 0.5 mL) was added dropwise and was stirred at 0 °C for 2 h. The reaction mixture was allowed to come to room temperature and continued stirring for 24 h. Reaction was monitored by HPLC. After completion of the reaction, DMF was removed under high vacuum and the blue residue was purified by flash column chromatography using a Combiflash separating system. The desired fractions were collected and lyophilized to get the FR-BODIPY as a dark bluish green solid (8mg, 89% yield) which was characterized by standard procedures. The obtained powder was subsequently complexed with defatted BSA as described elsewhere (Martin and Pagano, 1994).

[0079] Cell labelling: BODIPY-lac-Cer and BODIPY-TR-Cer complexed to bovine serum albumin, and 10kDa Dextran-Alexa546 and -Alexa647 were from Molecular Probes. BODIPY-lac-Cer and BODIPY-TR-Cer were incubated on cells at 5µM in 4°C Hank's Buffered Salt Solution (HBSS)/10mM HEPES for 30min; BODIPY650/665-lac-Cer was incubated at 5µM in 25°C HBSS/10mM HEPES for 30min (shorter incubation times did not give effective labelling); Dextran were incubated at 0.5mg/mL for 5 min at 25°C. After labelling, cells were washed three to five times in HBSS/10mM HEPES, followed by Schneider's medium, supplemented with 1% (v/v) OxyFluor (Oxyrase Inc., USA), for imaging.

[0080] Transfection: Transfections LAMP-GFP, MannosidaseII-GFP, and Rab11-GFP with were carried out using CellFectin lipid reagent (Invitrogen, USA) per manufacturer's instructions.

[0081] Cholesterol depletion/overload: For cholesterol depletion, MannosidaseII-GFP (see Bard et al, 2006) or LAMP-GFP (see Pulipparacharuvil et al, 2005) transfected cells were incubated with 10mM methyl- β -cyclodextrin (M β CD) (Sigma-Aldrich) at 25°C for 30min in serum-free medium, before labelling. After labelling, cells were further incubated in serum-free medium. For cholesterol excess,

10mM M β CD-cholesterol-complexes were prepared as in Klein, et al (Klein et al., 1995) and incubated with cells at 25°C for 30min before labelling. After treatment cells were further incubated in complete medium with serum.

[0082] Microscopy and co-localization studies: Confocal microscopy was carried out using a Leica SP2 microscope with a 63X/0.9NA water dipping lens. For double-labelling experiments, control experiments were carried out using the single fluorophores, to verify that there was no crosstalk between the emission channels.

[0083] For co-localization experiments all images in a given experiment were taken and processed identically. Co-localization values were calculated for at least 15 cells at each time point, using the algorithm from Costes *et al.* (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. Rasband) and "BG Subtraction from ROI" (by M. Cammer and T. Collins). ROI was set such that visible plasma membrane staining was not analyzed.

[0084] Results and Discussion

[0085] Lactosyl-Ceramide is taken up in an endocytic pathway by Drosophila neurons: The uptake and trafficking of fluorescently labelled analogs of the simple glycosphingolipid (GSL) lactosyl-Ceramide (lac-Cer) in a Drosophila larval brain-derived neuronal cell line, DL-DmBG2-c6 (hereafter called c6) (Ui, 1994) was studied. Flies produce GSLs, but since their content and structure are somewhat 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 (Choudhury et al., 2002; Choudhury et al., 2004; Puri et al., 2001; Sharma et al., 2003; Singh et al., 2003), and in fact, these two lipid classes are known to traffic interdependently (Mukherjee and Maxfield, 2004; Puri et al., 2003; Sharma et al., 2004; Sillence et al., 2002; te Vrugte et al., 2004).

[0086] 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, B). Fluorescent BODIPY-FL-tagged lac-Cer (FL-lac-Cer; Invitrogen) and

gradually becomes incorporated into mobile endocytic vesicles 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 far red-(FR)BODIPY-tagged lac-Cer (see Materials and Methods for synthesis of this label), 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.

[0087] These early lac-Cer carrying vesicles may be sorting endosomes that lac-Cer traverses en route to Golgi and other compartments, since the FR-Lac-Cer colocalizes with LAMP-GFP (Pulipparacharuvil et al., 2005) at a peak value of $tM_{lac-Cer} \approx 30\%$ after 30 min of chase time (Figure 1C; colocalization was based on the Manders algorithm, using Costes thresholding) (Costes et al., 2004; Manders, 1993)). At this time, a fluid phase marker of endocytic uptake, 10kDa 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, some of which may be mildly acidified (pH 5.9-6) sorting endosomes (Hunziker and Geuze, 1996).

[0088] From the quantitative colocalization studies over time, it was concluded that lac-Cer traffics primarily through sorting endosomes to the recycling endosomal compartment, and the remainder is localized to the Golgi and to lysosomes. A small proportion remains in vesicles that are presumed to be LAMP-GFP positive sorting endosomes. This is similar to what was reported in fibroblasts (Puri et al., 2001; Sharma et al., 2003), but the present findings differ in that the Golgi contribution is much less significant than that of recycling endosomes. Previously, it was reported that lac-Cer acted as a marker of the Golgi compartment, with a minor early contribution from recycling endosomes, which cycle the lipid back to the plasma membrane (Sharma et al., 2003).

[0089] It was also observed that a non-glycosylated fluorescent sphingolipid, FL-BODIPY- or TR-BODIPY-Ceramide (FL- or TR-Cer), is taken up into mobile endosomes as is the glycosylated FL-lac-Cer, and exhibits the interesting property of trafficking in tubulovesicular compartments that move along the length of neuronal processes. TR-Cer also showed some colocalization with dextran and lysotracker, although this was not quantified (Figure 1D).

[0090] Lac Cer is only partially Golgi associated: Since FL-lac-Cer was characterized as a marker of the Golgi compartment in human fibroblasts, testing for colocalization between FR-lac-Cer and the transfected *Drosophila* Golgi marker MannosidaseII-GFP (MannII-GFP)(Bard et al., 2006); Figure 2A, B) was conducted. Starting from less than 10% colocalization at 30', colocalization increased to 20% at 1h, and thereafter hovered near 20% up to 4h of chasing time (Figure 2B). This is significantly lower than would be expected for an exclusively Golgi marker. Thus, testing was done for the presence of FR-lac-Cer in other compartments. Colocalization with transfected LAMP-GFP, as mentioned above, peaked at ~30% at 30' in presumptive sorting endosomes, and then fell over the next hour of trafficking time 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 4h of chasing time (Figure 1B).

[0091] Lac-Cer can “hijack” the trafficking route of an endocytic cargo: Earlier studies by Pagano et al showed that lac-Cer stimulates caveolar uptake from the plasma membrane (Sharma et al., 2004). The present experiments reveal a novel effect, distinct from simple stimulation of uptake, whereby lac-Cer and another endocytic cargo, Dextran. This phenomenon is proposed to be called “hijacking” because it involves the re-routing of the trafficking of one agent by the other. Re-routing through a novel pathway would not be expected if lac-Cer were merely stimulating endocytosis, carrying Dextran with it.

[0092] In this case, 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), but surprisingly, they show almost complete colocalization when incubated directly one after the other. In contrast, their trafficking is almost entirely separate when incubated independently.

[0093] Sequential labeling of lac-Cer then Dextran (Lac-Cer pulse, 3h chase, Dextran pulse, 2h chase) or conversely, Dextran then lac-Cer (Dextran pulse, 2h chase, Lac-Cer pulse, 3h chase) both resulted in nearly no colocalization (Figure 3B, C). This was not surprising, since lac-Cer is reported to be predominantly Golgi

localized (Puri et al., 2001), and Dextran is predominantly lysosomal. Unexpectedly, however, lac-Cer and Dextran traffic completely together when added simultaneously ($tM \approx 80\%$; Figure 3A, B). This is unlikely to be due to direct binding between the two labels, since the 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 3E).

[0094] In order to find out what the new routes of the lac-Cer + Dextran combination are, a time course colocalizations with lac-Cer + Dextran in the presence of either a Golgi or a lysosomal reference marker was conducted, and colocalization of total lac-Cer intensity with the reference marker ($tM_{lac-Cer}$) was calculated. In each case, lac-Cer colocalized completely with pulsed Dextran (as seen in Figure 3A), but comparison with the late endolysosomal marker LAMP-GFP showed that lac-Cer diverts Dextran away from endolysosomes (compare green line with dashed black line; Figure 4A). 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 4B). The difference between the colocalization curves with LAMP-GFP vs. overnight Dextran is not unexpected, since LAMP-GFP is not strictly confined to lysosomes, and these two labels colocalize with each other only to $\sim 40\%$ (Figure 3C).

[0095] Lac-Cer's own itinerary is also slightly altered in the presence of Dextran (compare red lines with dashed black lines in Figure 4A-C). In particular, lac-Cer + Dextran follows a slower route toward the Golgi than lac-Cer alone (Figure 4C). Lac-Cer + Dextran appears to arrive late and persist at a slightly higher than normal level in endolysosomes labelled with LAMP-GFP (Figure 4A), while it is suppressed in lysosomes labeled with Dextran overnight (Figure 4B).

[0096] Thus, lac-Cer interacting with a ligand directs that ligand to an alternate intracellular path. As shown here, Dextran, which ordinarily follows an endosome $\rightarrow$ late endosome $\rightarrow$ lysosome pathway, is re-routed completely away from late endosomes/lysosomes when lac-Cer is also present. Lac-Cer is slowed in its progression toward Golgi in the presence of Dextran.

[0097] Lac-Cer trafficking to Golgi vs. lysosomal pathways 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 Niemann-Pick type C (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; Choudhury et al., 2004). Similarly, aberrant trafficking of GSL to lysosomes was found in a model of Gaucher disease (Sillence et al., 2002). Therefore, it was addressed here whether trafficking of GSLs in fly neurons is similarly affected to that in diseased human cells, under analogous conditions of lipid storage.

[0098] When cells are overloaded with cholesterol (see Materials & Methods), trafficking to endolysosomes is strongly increased at the expense of Golgi-directed trafficking (Figure 5C, D). The initial uptake pathway, presumably through early and sorting endosomes, is not perturbed, but becomes non-coincident at the point of divergence between the Golgi and endolysosomal pathways (~60 min) (see graphs in Figure 5C, D).

[0099] Under conditions of cholesterol depletion by incubation with methyl- β -cyclodextrin (M β CD), the converse effect is observed, with respect to Golgi trafficking: lac-Cer trafficking to Golgi appears to be initially slower than normal, until 2h, and then shows an enhanced peak at 3h (Figure 5A). Interestingly, this level returns to a similar baseline as in the control cells after 4h. This would fit with disruption of a cholesterol-dependent mechanism of uptake from the plasma membrane, and subsequent shunting of more lac-Cer through a transient Golgi pathway, rather than other possible itineraries in recycling or sorting endosomal compartments. Cholesterol depletion increases lac-Cer trafficking to endolysosomes to a similar degree as cholesterol excess (compare Figure 5B). Quantification of cholesterol levels in extracts of M β CD-treated c6 neurons showed a 45-50% reduction of cholesterol, using the Amplex RedTM cholesterol quantification kit (Invitrogen).

[00100] All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. The

citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

[00101] As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Unless defined otherwise all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs.

[00102] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

References

- Bard, F., L. Casano, A. Mallabiabarrena, E. Wallace, K. Saito, H. Kitayama, G. Guizzunti, Y. Hu, F. Wendler, R. Dasgupta, N. Perrimon, and V. Malhotra. 2006. Functional genomics reveals genes involved in protein secretion and Golgi organization. *Nature*. 439:604-7.
- Choudhury, A., M. Dominguez, V. Puri, D.K. Sharma, K. Narita, C.L. Wheatley, D.L. Marks, and R.E. Pagano. 2002. Rab proteins mediate Golgi transport of caveola-internalized glycosphingolipids and correct lipid trafficking in Niemann-Pick C cells 10.1172/JCI200215420. *J. Clin. Invest.* 109:1541-1550.
- Choudhury, A., D.K. Sharma, D.L. Marks, and R.E. Pagano. 2004. Elevated endosomal cholesterol levels in Niemann-Pick cells inhibit rab4 and perturb membrane recycling. *Mol Biol Cell*. 15:4500-11.
- Costes, S.V., D. Daelemans, E.H. Cho, Z. Dobbin, G. Pavlakis, and S. Lockett. 2004. Automatic and quantitative measurement of protein-protein colocalization in live cells. *Biophys J*. 86:3993-4003.
- Cutler, R.G., J. Kelly, K. Storie, W.A. Pedersen, A. Tammara, K. Hatanpaa, J.C. Troncoso, and M.P. Mattson. 2004. Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's disease. *PNAS*. 101:2070-2075.
- Huang, X., K. Suyama, J. Buchanan, A.J. Zhu, and M.P. Scott. 2005. A *Drosophila* model of the Niemann-Pick type C lysosome storage disease: *dnpc1a* is required for molting and sterol homeostasis 10.1242/dev.02079. *Development*. 132:5115-5124.
- Hunziker, W., and H.J. Geuze. 1996. Intracellular trafficking of lysosomal membrane proteins. *Bioessays*. 18:379-89.

- Klein, U., G. Gimpl, and F. Fahrenholz. 1995. Alteration of the myometrial plasma membrane cholesterol content with beta-cyclodextrin modulates the binding affinity of the oxytocin receptor. *Biochemistry*. 34:13784-93.
- Manders, E.M.M., Verbeek, F.J., Aten, J.A. 1993. Measurement of co-localization of objects in dual-color confocal images. *Journal of Microscopy*. 169:375-382.
- Marks, D.L., and R.E. Pagano. 2002. Endocytosis and sorting of glycosphingolipids in sphingolipid storage disease. *Trends Cell Biol*. 12:605-13.
- Martin, O.C., and R.E. Pagano. 1994. Internalization and sorting of a fluorescent analogue of glucosylceramide to the Golgi apparatus of human skin fibroblasts: utilization of endocytic and nonendocytic transport mechanisms. *J Cell Biol*. 125:769-81.
- Maxfield, F.R., and I. Tabas. 2005. Role of cholesterol and lipid organization in disease. *Nature*. 438:612-21.
- Min, K.T., and S. Benzer. 1997. Spongecake and eggroll: two hereditary diseases in *Drosophila* resemble patterns of human brain degeneration. *Curr Biol*. 7:885-8.
- Mukherjee, S., and F.R. Maxfield. 2004. Lipid and cholesterol trafficking in NPC. *Biochim Biophys Acta*. 1685:28-37.
- Myllykangas, L., J. Tyynela, A. Page-McCaw, G.M. Rubin, M.J. Haltia, and M.B. Feany. 2005. Cathepsin D-deficient *Drosophila* recapitulate the key features of neuronal ceroid lipofuscinoses. *Neurobiol Dis*. 19:194-9.
- Nakano, Y., K. Fujitani, J. Kurihara, J. Ragan, K. Usui-Aoki, L. Shimoda, T. Lukacsovich, K. Suzuki, M. Sezaki, Y. Sano, R. Ueda, W. Awano, M. Kaneda, M. Umeda, and D. Yamamoto. 2001. Mutations in the novel membrane protein spinster interfere with programmed cell death and cause neural degeneration in *Drosophila melanogaster*. *Mol Cell Biol*. 21:3775-88.
- Pagano, R.E. 2003. Endocytic trafficking of glycosphingolipids in sphingolipid storage diseases. *Philos Trans R Soc Lond B Biol Sci*. 358:885-91.
- Puglielli, L., R.E. Tanzi, and D.M. Kovacs. 2003. Alzheimer's disease: the cholesterol connection. 6:345-351.
- Pulipparacharuvil, S., M.A. Akbar, S. Ray, E.A. Sevrioukov, A.S. Haberman, J. Rohrer, and H. Kramer. 2005. *Drosophila* Vps16A is required for trafficking to lysosomes and biogenesis of pigment granules 10.1242/jcs.02502. *J Cell Sci*. 118:3663-3673.
- Puri, V., J.R. Jefferson, R.D. Singh, C.L. Wheatley, D.L. Marks, and R.E. Pagano. 2003. Sphingolipid storage induces accumulation of intracellular cholesterol by stimulating SREBP-1 cleavage. *J Biol Chem*. 278:20961-70.
- Puri, V., R. Watanabe, M. Dominguez, X. Sun, C.L. Wheatley, D.L. Marks, and R.E. Pagano. 1999. Cholesterol modulates membrane traffic along the endocytic pathway in sphingolipid-storage diseases. *Nat Cell Biol*. 1:386-8.
- Puri, V., R. Watanabe, R.D. Singh, M. Dominguez, J.C. Brown, C.L. Wheatley, D.L. Marks, and R.E. Pagano. 2001. Clathrin-dependent and -independent internalization of plasma membrane sphingolipids initiates two Golgi targeting pathways. *J Cell Biol*. 154:535-47.
- Sabharanjak, S., P. Sharma, R.G. Parton, and S. Mayor. 2002. GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Dev Cell*. 2:411-23.
- Seppo, A., M. Moreland, H. Schweingruber, and M. Tiemeyer. 2000. Zwitterionic and acidic glycosphingolipids of the *Drosophila melanogaster* embryo. *Eur J Biochem*. 267:3549-58.

- Sharma, D.K., J.C. Brown, A. Choudhury, T.E. Peterson, E. Holicky, D.L. Marks, R. Simari, R.G. Parton, and R.E. Pagano. 2004. Selective stimulation of caveolar endocytosis by glycosphingolipids and cholesterol. *Mol Biol Cell*. 15:3114-22.
- Sharma, D.K., A. Choudhury, R.D. Singh, C.L. Wheatley, D.L. Marks, and R.E. Pagano. 2003. Glycosphingolipids internalized via caveolar-related endocytosis rapidly merge with the clathrin pathway in early endosomes and form microdomains for recycling. *J Biol Chem*. 278:7564-72.
- Sillence, D.J., V. Puri, D.L. Marks, T.D. Butters, R.A. Dwek, R.E. Pagano, and F.M. Platt. 2002. Glucosylceramide modulates membrane traffic along the endocytic pathway. *J Lipid Res*. 43:1837-45.
- Singh, R.D., V. Puri, J.T. Valiyaveetil, D.L. Marks, R. Bittman, and R.E. Pagano. 2003. Selective caveolin-1-dependent endocytosis of glycosphingolipids. *Mol Biol Cell*. 14:3254-65.
- Sriram, V., K.S. Krishnan, and S. Mayor. 2003. deep-orange and carnation define distinct stages in late endosomal biogenesis in *Drosophila melanogaster*. *J Cell Biol*. 161:593-607.
- Sweeney, S.T., and G.W. Davis. 2002. Unrestricted synaptic growth in spinster-a late endosomal protein implicated in TGF-beta-mediated synaptic growth regulation. *Neuron*. 36:403-16.
- te Vrugte, D., E. Lloyd-Evans, R.J. Veldman, D.C. Neville, R.A. Dwek, F.M. Platt, W.J. van Blitterswijk, and D.J. Sillence. 2004. Accumulation of glycosphingolipids in Niemann-Pick C disease disrupts endosomal transport. *J Biol Chem*. 279:26167-75.
- Ui, K., Nishihara, S., Sakuma, M., Togashi, S., Ueda, R., Miyata, Y., Miyake, T. 1994. Newly established cell lines from *Drosophila* larval CNS express neural specific characteristics. *In Vitro Cell Dev Biol Anim*. 30A:209-216.
- Wolozin, B. 2004. Cholesterol and the biology of Alzheimer's disease. *Neuron*. 41:7-10.

WHAT IS CLAIMED IS:

1. A method of delivering an endocytic cargo to a cellular compartment comprising contacting a glycosphingolipid and the endocytic cargo with the external surface of a cell so that the glycosphingolipid and the endocytic cargo are taken up into the cell and targeted to a non-lysosomal compartment.
2. The method of claim 1 wherein the glycosphingolipid comprises lactosyl ceramide.
3. The method of claim 1 or claim 2 wherein the cell is a cell in culture.
4. The method of claim 1 or claim 2 wherein the cell is an explanted cell.
5. The method of claim 1 or claim 2 wherein the cell is in vivo.
6. The method of any one of claims 1 to 5 wherein the glycosphingolipid comprises a first detectable label.
7. The method of claim 6 wherein the first detectable label comprises a fluorescent group.
8. The method of claim 7 wherein the first detectable label comprises BODIPY-Far Red 650/665.
9. The method of any one of claims 6 to 8, the method further comprising detecting the first detectable label.
10. The method of any one of claims 1 to 9 wherein the endocytic cargo comprises a second detectable label.
11. The method of claim 10 wherein the second detectable label comprises a fluorescent group.
12. The method of claim 11 wherein the second detectable label comprises BODIPY-Far Red 650/665.
13. The method of any one of claims 10 to 12, the method further comprising detecting the second detectable label.

14. The method of claim 9 or claim 13 wherein at least one of the first detectable label and the second detectable label comprises a fluorescent group and the detecting comprises visualizing the fluorescent group using fluorescence microscopy.
15. The method of any one of claims 6 to 14 wherein in the cell is a cell from a subject for diagnosis of a sphingolipid related disorder, the method further comprising comparing the glycosphingolipid trafficking pattern in the cell with the glycosphingolipid trafficking pattern observed for a healthy cell.
16. The method of any one of claims 1 to 15 wherein the endocytic cargo comprises a therapeutic agent.
17. The method of claim 16 wherein the therapeutic agent comprises a drug, an antibiotic, a hormone, a cellular factor, a nucleic acid molecule or a polypeptide.
18. The method of claim 16 or claim 17 wherein an effective amount of the endocytic cargo comprising the therapeutic agent is contacted with the external surface of the cell of a subject in need of treatment of a sphingolipid related disorder or a disorder in which the endocytic cargo comprising a therapeutic agent is desired to be re-directed within a cell.
19. The method of claim 15 or claim 18 wherein the sphingolipid related disorder is a neurodegenerative disorder.
20. The method of claim 19 wherein the neurodegenerative disorder is Alzheimer's disease.