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Characterization and application of a disease-cell model for a neurodegenerative lysosomal disease

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Abstract

Disease-cell models that recapitulate specific molecular phenotypes are essential for the investigation of molecular pathogenesis of neurodegenerative diseases including lysosomal storage diseases (LSDs) with predominant neurological manifestations. Herein we report the development and characterization of a cell model for a rapid neurodegenerative LSDs, globoid-cell leukodystrophy (GLD), mostly known as Krabbe disease. GLD is caused by the deficiency of β -galactocerebrosidase (GALC), a lysosomal enzyme that hydrolysis two glycosphingolipids, psychosine and galactosylceramide. Unfortunately, the available culture fibroblasts from GLD patients consist in a limited research tool as these cells fail to accumulate psychosine, the central pathogenic glycosphingolipid in this LSD that results in severe demyelination. Firstly, we obtained brain samples from the *Twitcher* (*Twi*) mice ($GALC^{twi/twi}$), the natural mouse model with GALC deficiency. We immortalized the primary neuroglial cultured cells with SV40 large T antigen, generating the 145M-*Twi* and the 145C-*Wt* cell lines from the *Twi* and control mice, respectively. Both cell lines expressed specific oligodendrocyte markers including A2B5 and GalC. The 145M-*Twi* cells showed biochemical and cellular disturbances related to GLD neuropathogenesis including remarkable caspase-3 activation, release of cytochrome C into the cytosol and expansion of the lysosomal compartment. Under treatment with glycosphingolipids, 145M-*Twi* cells showed increased LC3B levels, a marker of autophagy. Using LC-MS/MS method we developed, the 145M-*Twi* cells showed significantly higher levels of psychosine. The

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145M-*Twi* and 145C-*Wt* lines allowed the development of a robust throughput LC-MS/MS assay to measure cellular psychosine levels. In this throughput assay, L-cycloserine showed to significantly reduce the 145M-*Twi* cellular levels of psychosine. The established 145M-*Twi* cells is a powerful research tool to investigate neurologically relevant pathogenic pathways as well as to develop primary screening assays for the identification of therapeutic agents for GLD and potentially other glycosphingolipid disorders.

Keywords

β -galactocerebrosidase; Krabbe Disease; globoid-cell leukodystrophy; psychosine; LC-MS/MS

1. Introduction

Glycosphingolipids are complex lipids with broad structural variety and cell-dependent expression, assuming major roles in physiological functions related to membrane structure and intracellular signaling [1]. These lipids may participate in the various pathogenic disease processes [2–5]. Given that glycosphingolipids are highly expressed in the central nervous system [1], disturbances in their degradation/biosynthetic pathways are associated with numerous neurodegenerative disorders [6]. Krabbe disease (MIM#245200), originally described as globoid-cell leukodystrophy (GLD), is a classical example in which a glycosphingolipid (psychosine) is an important pathogenic molecule in the neurodegenerative process [7]. The exact mechanism of psychosine cytotoxicity is still unclear. Recent studies demonstrated that psychosine and its enantiomer have equal or greater cytotoxicity by localizing to lipid rafts, disturbing plasma membrane integrity and inhibiting protein kinase C translocation to the plasma membrane [8]. GLD is an autosomal recessive disease caused by the deficiency of β -galactocerebrosidase (GALC; EC 3.2.1.46), a lysosomal hydrolase that has galactosylceramide and psychosine as natural substrates [9]. The most common clinical form of GLD is characterized by progressive demyelination resulting in hyperirritability, hypotonia, intractable seizures and ultimately death within the first 1–2 years of age [10].

During myelination, uridine diphosphate-galactose:ceramide galactosyltransferase catalyzes the galactosylation of sphingosine and ceramide producing psychosine (galactosylsphingosine) and galactosylceramide, respectively [11]. Psychosine is promptly metabolized by GALC into sphingosine, maintaining low physiological levels of psychosine [7, 11]. In brain specimens from GLD patients however, apart from the infiltration of multinucleated PAS-positive macrophages, the “globoid-cells” [7], substantial neural cell loss is observed [12, 13]. These neuropathological findings are explained by the rapid effects of psychosine which, at elevated concentrations, becomes highly cytotoxic [14], in particular to oligodendrocytes, resulting in progressive demyelination [7]. This rapid neurodegenerative process results in, less evident, and even reduced, cellular storage, a hallmark cellular finding classically seen in several other lysosomal disorders [7].

Unfortunately, the sphingolipid and glycosphingolipid metabolism is poorly expressed in most cells other than those of neural origin. However, due to the recent advances on the technology of mass spectrometry (MS), measurement of certain glycosphingolipids is feasible in easily available specimens [15]. The non-availability of established neural cells has limited the understanding of neuropathogenic mechanisms in GLD as well as other lysosomal storage diseases (LSDs). Based on the major role of elevated psychosine levels in the neuropathogenesis of GLD, it is conceivable that the development of a neural disease-cell model will allow the identification of neurologically relevant pathogenic cascades,

which can be used as targets in the discovery of therapies for this rapidly neurodegenerative disease.

Here we describe the development, characterization and potential application of a neurologically relevant cell line from the *Twitcher* (*Twi*) mouse, the classical GLD model with a naturally-occurring mutation in *GALC* gene [16]. Using a newly developed liquid chromatography MS (LC-MS/MS) assay, the 145M-*Twi* cells showed increased psychosine levels. In the 145M-*Twi* cells, we report novel molecular observations, which have direct implications in the pathogenesis of GLD. In addition, we demonstrate the potential of these cells to be utilized as a research tool for developing screening assays to allow the identification of therapeutic agents for this disorder.

2. Material and Methods

Cell Culture and Transformation

Primary cortical cells were obtained from dissected brain cortices from 5 pups at first day of life from *Twi* C57BL/6J mouse (*GALC*^{wt/wt}) and 5 pups from a CB57BL/6J control mouse (*GALC*^{wt/wt}). Primary cortical neuroglial cells were cultured using DMEM/F-12 medium with 1% penicillin/streptomycin and 10% dialyzed fetal bovine serum (Benchmark FBS). Cells were cultured using traditional incubators with 5% CO₂ at 37°C. Cultured primary neuroglial cells were transformed using pSV3-neo plasmid containing the simian virus (SV) 40 large T cell antigen as previously described [17]. Two immortalized cell lines were established: 145M-*Twi*, transformed cells from the *Twi* mice; 145C-*Wt*, transformed cells from the control mice. Further information is detailed in Supplementary data.

GALC and caspase-3 enzymatic assays

The 145M-*Twi* and 145C-*Wt* cells were cultured in 75 cm² flasks for obtaining lysates for GALC assay. On average 10 µg of protein lysate was used per assay. The GALC assays were performed with specific synthetic fluorescent 6-hexadecanoylamino-4-methylumbelliferyl-β-D-galactoside (HMUβGal) following procedures previously described [18]. After stopping assay, 260 µL aliquots from the total solution were transferred to 96-well plates for reading in a fluorescence plate reader at wavelengths of HMU ($\lambda_{\text{excitation}} = 404 \text{ nm}$; $\lambda_{\text{emission}} = 406 \text{ nm}$). Assays were run in quadruplicate samples for *Twitcher* and control mice cell pellets. Caspase-3 assays were performed with 20 µM concentrations of *N*-acetyl-DEVD 7-maino-4-methylcoumarin (Ac-DEDV-AMC) synthetic fluorescent substrate for one hour as previously described [19]. 145M-*Twi* and 145C-*Wt* from *Twitcher* and control mice, respectively, were cultured in 6-well plates with wells treated with 15 and 30 µM of psychosine (purity >98%; Matreya LLC) and glucosyl-sphingosine (purity >98%; Matreya LLC). After 24hs, these cells were washed with PBS and harvested with 0.05% trypsin solution. After centrifugation and aspiration of PBS, cell lysate preparation and assay procedures were performed as per manufacturing protocol and as previously described [19].

Immunocytochemistry assays

First, cells were grown to 70–80% confluence on round coverslips, washed with PBS and fixed with 4% paraformaldehyde for 15 min, following by blocking with PBS 10% goat serum for 30 min. After washing twice with PBS, cells were incubated in the presence of anti-A2B5 (1:400 in PBS 1% goat serum), anti-GalC (1:200 in PBS 1% goat serum), GFAP (1:200 in PBS 1% goat serum) and MAP2 (1:1,000 in PBS 1% goat serum), or antibodies at room temperature for 2 h. The cells were then immunostained with suitable secondary antibodies (1:200; Molecular Probes – Invitrogen Inc.) at room temperature for 1 h. Using confocal laser scanning microscopy on the Zeiss LSM 510, images were taken with 100 × 1.4 numerical apertures (NA) and 63 × 1.4 NA Apochromat objective (Zeiss). For staining

for cytochrome C and LysoTracker, monoclonal antibody was purchased from BD Pharmingen™ (clone 6H2.B4) and LysoTracker Red (DND-99) probe was purchased from Molecular Probes – Invitrogen Inc.. Cells were cultured in 70–80% confluence over coverlips and exposed to 0.5 μ M of LysoTracker Red for 30 min before fixation step with formaldehyde 2% in PBS also for 30 min. For these immunofluorescence assays, the GalC and A2B5, monoclonal antibodies were purchased from Millipore Corp. (Billerica, MA). For LC3-B confocal immunofluorescence assays, LC3B (D11) XP Rabbit monoclonal antibody was purchased from Cell Signaling™ and used at 1:200 dilution. Secondary antibodies Alexa Fluor® F(ab)₂ against mouse and were purchased Invitrogen™ (Life Tech, Grand Island, NY). The blocking procedure (PBS in 10% goat serum), primary and secondary antibody staining were as described above.

Western Blott

Immunoblot analysis for cleaved caspase-3 was performed using standard procedures [20]. 145M-*Tw*i and 145C-*W*t from *Tw*i and control mice, respectively, were cultured to confluence and treated with different concentrations of psychosine 7.5–30 μ M and glucosylsphingosine for 24hs and harvested and lysed in standard lysis buffer containing a protease inhibitor cocktail (Thermo-Fisher Inc.). Anti- α -tubulin mouse (DM1A) antibody (1:3,000) was used as a loading control and purchased by EMD Milipore™. Secondary HRP-antibody used was rabbit anti-mouse IgG at 1:10,000. Blots were developed using chemiluminescent substrate using cooled CCD camera coupled with software from Alpha Innotech Corp. (Amersham Biosciences).

Liquid chromatography mass spectrometry (LC-MS/MS)

Samples were analyzed by LC-MS/MS on the Applied Biosystems (SCIEX) API4000 (Applied Biosystems, Foster City, CA) coupled with Agilent 1100 autosampler. The LC-MS/MS injections were 8 μ L from cellular lipid extracts. The LC column used was C8 X-Terra MS, 125 Å, 3.5 μ m, 1 $\times$ 50mm. The total run time was 2 minutes. Given that the both retention time for the psychosine and 1-acetyl-psychosine were < 1 min, the run time was further reduced to 1 min. Table S1 (Supplementary data) describes details of the psychosine LC-MS/MS conditions as well as the mobile phase A (MPA) and mobile phase B (MPB). The MRM transitions monitored were m/z 462 to 264 for psychosine and m/z 504 to 264 for 1-acetyl-psychosine, the ISD (Fig. 2a). For measurements of psychosine levels, areas under the peaks in the chromatograms were measured for both psychosine and 1-acetyl-psychosine (ISD). The pmoles of psychosine were calculated based on the ISD present in each sample 19.8 pmoles/100 μ L. The initial determination of RTs of psychosine and 1-cetyl-psychosine (Fig. 2a), methanol solutions with different concentrations of these standards of psychosine and 1-acetyl-psychosine (both with purity >98%; Matreya LLC) were injected at 8 μ L volume into the API4000 LC-MS/MS system. In terms of the LC-MS/MS validation assay, BEH column was used with a running time of 30 min. Two ACQUITY UPLC BEH Glycan (Waters Inc.) columns, as described above (1.7 μ m, 2.1 $\times$ 100mm and 1.7 μ m, 2.1 $\times$ 150mm), were coupled, being first 2.1 $\times$ 150mm and the second 2.1 $\times$ 100mm. Details on the conditions were detailed in Table S2 (Supplementary data).

Brain specimens for psychosine LC-MS/MS analysis

Brain samples from one patient manifesting the infantile and another with the late onset GLD were obtained from NICHD Brain and Tissue Bank for Developmental Disorders, University of Maryland, Baltimore, MD. The brain specimens from GLD patients comprise of frozen samples from each brain region were weighted to determine the dry weight using high-precision scales to standardize the measurements of glycosphingolipids levels in

further assays. The lipid extraction was performed using chloroform:methanol (2:1, v/v) as previously described [21].

Lipid extractions

For the lipid extraction from cells, lysates from 145M-*Tw*i and 145C-*W*t, derived from single clones of SV40-transformed primary neuroglial cells, received a volume of 150 μ L of methanol (polar solvent) with ISD, 1-acetyl-psychosine (198.5 nM) was used. After shaking tubes for 60 min (orbital shaker), and spinning tubes (13,000 rpm) for 2–3 minutes, lipid extract solutions were passed through individual centrifuge 2 mL-tube filter (0.22 μ polypropylene. A volume of 8 μ L of the filtered solution was directly injected into the API 4000. For cell cultured in 96-well plates, the 145M-*Tw*i and 145C-*W*t were cultured and then washed with PBS, polar solvent methanol with 0.5% acetic acid was dispensed directly into cells. Lipid extractions are amenable to be performed in 96-well plates [22]. A volume of 80 μ L of the methanol with 0.5% acetic acid solvent containing 1-acetyl-psychosine (198.5 nM), as internal standard, was efficient for psychosine extraction in the multi-well plate. After placing the solvent, plates were sealed with 96-well polypropylene pierceable round cap mat and agitated in a horizontal orbital shaker for 60 minutes. The extraction solution was then transferred to the filter plate AcroPrepTM Advance 96 (0.2 μ supor membrane) assembled to 96-well sample collection plate (1mL/well). The 96-well filter/sample collection assembly was then centrifuged at 1,400 rpm for 3 min. After the centrifugation step, the filter plate was discarded and the 96-well sample collection plate was sealed with a new 96-well polypropylene pierceable round cap mat. Plates were kept in -20°C before injections into the API 4000.

3. Results

Establishment and characterization of cultured 145M-*Tw*i cell lines from GLD mouse model

To develop an optimal disease cell-line that expresses glycosphingolipids and is representative of neurological pathogenic processes in GLD, we firstly established primary cultured neuroglial cells derived from cortices from *Tw*iher (*Tw*i) $\text{GALC}^{twi/twi}$ mice at day 1 of life [16, 23] (*Material and Methods*). The C57BL/6J *Tw*i carries a homozygous naturally-occurring nonsense mutation (c.1017G>A/W339X) resulting in a truncated and non-functional GALC with a clinical and neuropathological phenotype that recapitulates GLD [24]. We then successfully transformed the cortical primary neuroglial cells by transfecting the pSV3-neo plasmid containing the simian virus (SV) 40 large T-cell antigen. Subsequently, six clones from the SV-40 transformed (SV40T) cells were isolated and cultured separately (Fig. 1a). Primary neuroglial cells from the control mice, also C57BL/6J but with $\text{GALC}^{wt/wt}$, were also transformed following the same procedure. Two SV40T transformed cells were generated: the 145M-*Tw*i cell line derived from *Tw*i ($\text{GALC}^{twi/twi}$), and the 145C-*W*t derived from *W*t ($\text{GALC}^{wt/wt}$). In confocal cellular immunostaining assays, the transformed cells consistently expressed galactosylceramide (GalC) and A2B5, two classical oligodendrocyte markers (Fig. 1b). Interestingly, increased Galc signal was noted in the 145M-*Tw*i when compared to the 145C-*W*t controls (Fig. 1b, panels D and E). The neuronal specific marker MAP2 was absent in both 145M-*Tw*i and 145C-*W*t cell lines (Fig. 1b). These transformed cells expressing oligodendrocyte markers were named 145M-*Tw*i those from *Tw*i mice, and 145C-*W*t those from the control mice. Few GFAP-positive cells, an astrocyte-specific marker, were noted amongst the 145M-*Tw*i and 145C-*W*t cell lines (Fig. 1b). We confirmed the presence of the homozygous nonsense mutation in the *GALC* gene in the 145M-*Tw*i cells (Fig. 1c). Using the synthetic fluorescent substrate, HMu β Gal, the 145M-*Tw*i ($\text{GALC}^{twi/twi}$) showed significantly reduced GALC enzymatic activity in comparison to 145C-*W*t controls ($\text{GALC}^{wt/wt}$) (Fig. 1d). None of the clones showed

differences in terms of the biochemical, morphological and cell growth aspects assessed in the assays above.

Development of a LC-MS/MS assay for measuring cellular psychosine

To determine the cellular levels of psychosine on the 145-*Twi* and 145C-*Wt*, a specific LC-MS/MS method was developed. The LC-MS/MS is a powerful method for detection and quantification of a specific glycosphingolipid through Multiple Reaction Monitoring (MRM), where a metabolite is repetitively scanned for selected precursor-product ion pairs to enhance the sensitivity and specificity of the analysis [25]. We used a C8 reserve MS X-Terra column (125 Å, 3.5 µm, 1×50 mm), whose composition of rugged particles confers injection resistance to dimethyl sulfoxide (DMSO), a common utilized drug-library solvent. Additionally, the high-mass capacity and wide pH range of this column provide the required flexibility for the development of a throughput assay. LC-MS/MS assays were done in the AB SCIEX API 4000 (Table S1). Using psychosine standards (461.64 g/mol), we were able to determine a sensitive and consistent MRM for psychosine 462/264 (Fig. 2a). The psychosine analog, 1-acetyl-psychosine (503.68 g/mol), absent in biological specimens, proved to be a reliable internal standard (ISD) for this assay with MRM 504/264 and similar extraction recovery and retention time (RT) of 0.42 min (Fig. 2a). The carry-over of psychosine and 1-acetyl-psychosine varies from 0.1 to 1.6% and from 0.1 to 0.43%, respectively. Using this methodology, both psychosine and 1-acetyl-psychosine showed RTs < 1 min, which is encouraging for throughput assay development.

Initially, we examined psychosine levels in brain samples from patients affected with GLD, which were previously shown to have significant higher levels of psychosine [26] (Fig. 2b and 2c). Brain samples from one male patient manifesting the infantile GLD (early onset of symptoms and rapidly progressive disease who died at 5 months of age) and from another adult male patient with the late onset GLD (protracted disease course who died at 39 years of age) were used in this assay. The brain samples were obtained from the Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. The lipid extraction was performed in dried samples from the white matter of regions from each patient and controls as previously described [27]. The measurements of psychosine in GLD patient brains were significantly higher than controls (Fig. 2b and 2c). The highest levels of psychosine were present in the brain from the infantile GLD patient. The psychosine levels present in lipid extracts from the brain of the late onset GLD patient were significantly elevated but lower than those detected in the patient with the infantile and more severe form of GLD (Fig. 2b). Given the sensitivity of LC-MS/MS method, a small level of psychosine was detectable in brain samples from age-matched controls (Fig. 2b and 2c).

Cultured 145M-*Twi* and 145C-*Wt* cells were harvested ($\sim 3.2 \times 10^6$ cells) and underwent lipid extraction with methanol, a polar lipid solvent. 1-acetyl-psychosine (198.5 nM) was used as an ISD (*Material and Methods*). Envision throughput applications, nitrogen evaporation and the use of solid-phase extraction (SPE) columns were avoided. Lipid extraction solutions were directly filtered in individuals 0.22 µ-tube-filters. The 145M-*Twi* showed significantly elevated levels of psychosine when compared to the 145C-*Wt* control cells (Fig. 2d).

The 145M-*Twi* cells showed increased caspase-3 activation and altered cytochrome C distribution

To assess susceptibility to apoptosis, we measured the caspase-3 activity in both 145M-*Twi* and 145C-*Wt* cells before and after treatment with different concentrations of psychosine in culture media, and its isomer glucosylsphingosine, which also showed a degree of cytotoxicity in previous studies [28] (Fig. 3a). Using the Ac-DEVD-AMC, specific fluorescent substrate for caspase-3, the 145M-*Twi* showed increased caspase-3 activation,

which was significantly enhanced under 24h-treatment with different concentrations of psychosine and glucosylsphingosine (Fig. 3). Psychosine produced a more robust activation of caspase-3 when compared to the glucosylsphingosine (Fig. 3a). The 24h-treatment with glucosylsphingosine (15 and 30 μ M) produced 1.8 ± 0.6 and 2.1 ± 0.4 fold increases in the caspase-3 activity in 145M-*Twi* and 145C-*Wt* cells, respectively (Fig. 3a). Whereas, after the 24h-treatment with psychosine at the same concentrations, the activation of caspase-3 was 1.9 ± 1.2 fold in 145C-*Wt* cells and, 3.9 ± 1.6 fold in 145M-*Twi* cells ($p=0.04$). Even in the absence of exogenous complex lipids, 145M-*Twi* cells showed increased caspase-3 activation ($p=0.014$; Fig. 3a). Confirming the caspase-3 activation measured by specific enzymatic assays, the immunoblots showed increased levels of cleaved and activated caspase-3 in 145M-*Twi* cells in a dose-response manner (Fig. 3b). In addition, the 145M-*Twi* cells showed a larger lysosomal compartment, which was not evident in the 145C-*Wt* control cells (Fig. 3c). Under treatment with psychosine, expansion of lysosomal compartment was more pronounced in 145M-*Twi* cells (Fig. 3c). Interestingly, in the control 145C-*Wt* cells, the lysosomal compartment was not evident until cells were treated with psychosine (Fig. 3c). Another important observation is the pattern of cytochrome c (Cyto C) staining in 145M-*Twi* cells. In 145M-*Twi* cells treated with glucosylsphingosine, and especially psychosine, the long and string Cyto C pattern became punctuated and more disorganized, indicating fragmented mitochondria and release of Cyto C into the cytosol was observed. In control 145C-*Wt* cells also treated with the two glycosphingolipids, the Cyto C staining showed consistently the long and string Cyto C pattern, indicating normal and intact mitochondria (Fig. 3c). It is important to emphasize that both 145M-*Twi* and 145C-*Wt* have normal lysosomal glucocerebrosidase activity. In addition, the levels of both complex lipids used in the culture medium were higher than cellular endogenous levels.

Increased LC3B, an autophagy marker in 145M-*Twi* cells

Along with apoptosis, autophagy is another vacuolar stress-mechanism responsible for the removal of long-lived proteins and sequestration of cytoplasmic damaged organelles into double-membrane bound organelles known as autophagosomes, and their subsequent delivery to lysosomes for degradation and recycling. Additionally, autophagy also function as an “adaptive” cell survival mechanism to maintain the levels of nutrients and energy levels during periods of stress [29]. Autophagy has been shown to be involved in the pathogenesis of some LSDs [30]. To investigate whether the 145M-*Twi* cells demonstrate any alteration in autophagy, using confocal immunofluorescence, we evaluated the levels of cleaved LC3B, a phosphatidylethanolamine-protein which is the key marker for autophagy induction [31]. Autophagy was assessed in the presence of multiple concentrations of exogenous psychosine and glucosyl-sphingosine during 24h period. The 145M-*Twi* cells showed increased cleaved LC3B levels under treatment with psychosine, which was elevated in comparison to 145C-*Wt* controls (Fig. 4). In 145M-*Twi* cells, the cleaved LC3B autophagy protein is localized in lysosomes (Fig. 4). The cleaved LC3B levels were mildly increased under treatment with glucosylsphingosine at 7.5 and 15 μ M, but remarkably increased in 145M-*Twi* cells treated with correspondent concentrations of psychosine (Fig. 4).

Distinguishing of psychosine from glucosylsphingosine in 145M-*Twi* cells

Glucosylsphingosine is an isomer of psychosine (galactosylsphingosine) with identical molecular weight (461.64 g/mol), but with a different orientation of hydroxyl residue bound to C4 carbon of the hexagonal carbohydrate ring (Fig. 2a). To confirm that the LC-MS/MS short-run (<1 min) using C8 reverse MS column specifically detects and quantifies psychosine from the cell-lipid extracts, we measured psychosine from the same lipid extracts using the two coupled Bridged Ethyl Hybrid (BEH) Glycan columns (2.1 $\times$ 150mm, 1.7 μ and 2.1 $\times$ 100 mm, 1.7 μ - Waters Inc. - Table S2). With the BEH columns, which are commonly

applied to identify complex *N*- and *O*-linked structures composed of frequently similar and repeating sugar moieties [32], we showed a distinct separation of psychosine from glucosylsphingosine (Fig. 5a–c). In fact, lipid extracts from 145M-*Tw*i cells showed consistently an evident peak corresponding to psychosine, which was not present in the lipid extracts from 145C-*Wt* control cells (Fig. 5b and 5c). Similar results were obtained when analyzing extracted lipids from brain samples from GLD patients and controls (Fig. S1).

Development of throughput LC-MS/MS assay to measure cellular psychosine in 145M-*Tw*i

Based on the increased levels of psychosine in the 145M-*Tw*i cells, we evaluated the potential of these cells to be used in a throughput assay, which can be ultimately used in drug screen assays. The 145M-*Tw*i and 145C-*Wt* were cultured in 96-well plates ($32\text{--}34 \times 10^3$ cells/well) followed by lipid extractions. In order to increase feasibility for a throughput assay, methanol (99.5%) was used as the sole solvent adding a small percentage of acetic acid (0.5%) to maximize the extraction of polar sphingolipids as psychosine. This solvent allowed the lipid extraction to be performed directly in the 96-well polystyrene tissue culture plates. Using psychosine standards, the sensitivity analysis of the LC-MS/MS assay for psychosine was determined. The standard psychosine was diluted in the lipid extraction solvent in a broad range of concentrations with a final volume of 150 μL dispensed in each well of a 96-well collection plate. The LC-MS/MS method showed high sensitivity and remarkable linearity in both lower and higher psychosine concentrations (Fig. 6a). A volume of 80 μL per well of the methanol:acetic acid (99.5:0.5) solvent containing 1-acetyl-psychosine, as ISD (198.5 nM), resulted in efficient cellular psychosine extraction (Fig. 6b and Table S2).

Importantly, no nitrogen evaporation or SEP column steps were needed for sample preparation pre-injection. In order to assure the samples were clean prior to LC-MS/MS injection, a filter plate AcroPrepTM Advance 96 (0.2 μ) assembled to 96-well sample collection plate was used following “on-plate” cellular lipid extraction. Using the Agilent 1100 autosampler (Waters Inc.), samples from the 96-well collection plate were automatically injected at 8 μL /well (80 μL sample volume/well). To test the robustness of the assay in 96-well plates, an interleaved uniformity plate-assessment assay was performed (Fig. 6b). The 145M-*Tw*i, 145C-*Wt* and wells containing no cells, with only medium (background) were allocated in alternating column positions as previously described [33]. The assessment demonstrated feasibility and robustness of the throughput LC-MS/MS cellular psychosine assay (Fig. 6b–c). The assay-window ratio of psychosine levels in 145M-*Tw*i cells to controls are of $\sim 9\text{--}16$ with a *Z'* score factor of 0.40–0.57 (Table 1).

Using 145M-*Tw*i cells for a throughput LC-MS/MS cellular psychosine assay

To confirm the potential of the developed cell-based throughput LC-MS/MS assay to detect small molecules that reduce cellular psychosine, 145M-*Tw*i cells cultured in 96-well plates were treated with L-cycloserine, a small molecule previously shown to reduce psychosine levels *in vivo* [34]. At culture medium concentrations ranging from 50–250 μM over 9-day period, L-cycloserine significantly reduced psychosine levels in 145M-*Tw*i cells (Fig. 7). On the other hand, no alterations of the already small levels of psychosine in the 145C-*Wt* control cells were observed (Fig. 7).

4. Discussion

Globoid-cell leukodystrophy (GLD), mostly known as Krabbe disease, is typical representative of neurological LSDs. GLD is caused by the deficiency of GALC, which is a soluble lysosomal hydrolase that catalyzes the removal of galactose-containing glycosphingolipids including galactosylceramide and psychosine [35]. During myelination,

galactosylation of sphingosine produces psychosine, a reaction catalyzed by UDP-galactose:ceramide galactosyltransferase – known as the Cleland and Kennedy pathway [11]. Under physiological conditions, the produced psychosine is rapidly degraded by GALC, resulting in low and, almost undetectable, levels of this sphingolipid in brain of healthy individuals [7]. In the setting of GALC deficiency, however, psychosine levels are elevated in the brain of patients with GLD [26]. At high levels, psychosine becomes cytotoxic, particularly to oligodendrocytes, the myelin-forming cells, resulting in apoptosis and extensive demyelination classically observed in brain imaging studies of GLD patients. The degree of demyelination correlates with disease progression [7].

Most current therapies for LSDs have been limited to treatment of non-neurological symptoms. Few enzyme replacement treatment (ERT) agents are FDA-approved and used to successfully treat and control symptoms of some LSDs [36]. Unfortunately, being large molecular-weight molecules, ERT agents are unable to cross the blood-brain barrier (BBB). By inhibiting serine palmitoyltransferase, a key step in the biosynthesis sphingolipids, L-cycloserine showed to delay weight loss and prolong survival of the *Twi* mouse, the murine model of GLD [34, 37]. However, because of its detrimental effects secondary to the generalized sphingolipid deprivation, L-cycloserine has failed to reach clinical use. Therefore, a specific inhibitor of UDP-galactosylceramide transferase would be desirable. Hematopoietic stem cell transplantation (HSCT) is the treatment offered for the most severe and early-onset clinical form of GLD [38].

Although HSCT prevents the typical fulminant course of the infantile form of GLD, presymptomatic patients who receive HSCT eventually present with debilitating neurological symptoms, indicating disease progression [39]. The advent of newborn screening programs for GLD [40] and other LSDs urge the development of novel types of therapies that can tackle the neuronal pathogenesis and alter the natural disease course. Small molecules, which are more likely to cross the BBB and reach the affected neural cells, have become a more attractive approach.

The investigation of affected tissue and, especially with live cells obtained from *in vivo* models, has allowed the understanding of the pathogenic mechanisms of the disease. In the study of neurological diseases, the establishment of brain cell lines is important to understanding of mechanisms causing and contributing to the neurological processes. Because glycosphingolipids, as psychosine and several other sphingolipids, are poorly expressed in cells and tissues other than those originated from the nervous system, the 145M-*Twi* (GALC^{*twi/twi*}) several characteristics of an optimal cell-line for the study of different pathways related to the lysosomal dysfunction with implications in the neuropathogenesis of GLD [16]. To have sufficient cells for the experiments designed, we successfully transformed these cells using SV 40 large T-cell antigen. Post-transformation, six clones were selected from the 145M-*Twi* and 145C-*Wt*, the background control (Fig. 1a). Subsequently, these cells behaved as stable immortalized cells. Amongst the different isolated clones from each cell line, 145M-*Twi* and 145C-*Wt*, no differences were noted regarding the psychosine levels, morphology and rate of cell growth. Confocal cellular immunostaining showed that both 145M-*Twi* and 145C-*Wt* consistently express galactosylceramide (GalC) and A2B5, two classical oligodendrocyte markers (Fig. 1b). The 145M-*Twi* cell line showed increased accumulation of galactosylceramide as expected (Fig. 1b). No MAP2-positive cells were observed and a few GFAP-positive cells were noted (Fig. 1b). Several human and murine oligodendrocyte cell lines have been generated using SV40 large T antigen [41–45]. The morphology of the both 145M-*Twi* and 145C-*Wt* were similar to the immortalized oligodendrocytes 158N and 158JP, generated from the normal (wild type) and *jimpy* mice, which is an X-linked dysmyelination model carrying a A>G mutation at the splice site acceptor site of exon 5 of the *PLP/DM20* gene [45, 46]. The cellular

phenotypic characteristics, in addition to the elevated levels of psychosine detected in the 145M-*Twi* cells, suggest that both immortalized 145M-*Twi* and 145C-*Wt* have oligodendrocyte origin. In terms of cell maintenance, no extra growth factors were required to maintain these cells, which were cultured in regular DMEM/F-12 medium with fetal bovine serum (10%). A previous study reported the generation of spontaneously immortalized Schwann cells from *Twi* mice [47]. These previously described cells were distinct from the ones here described. Firstly, they were originated from peripheral nervous system, *i.e.* Schwann cells (TwS1) which were present in specimens collected from the dorsal root ganglia and peripheral nerves of 3-week-old *Twi* mice [47]. Secondly, these TwS1 cells require more laborious tissue culture procedures [48]. For these reasons, the immortalized 145M-*Twi* cells reported bring advantages from a standpoint of tissue culture procedures and being obtained directly from CNS.

The 145M-*Twi* cells showed several characteristics to be a disease-cell model for GLD. This cell line showed reduced GALC enzymatic activity and confirmed to carry the nonsense mutation in *GALC* gene (Fig. 1c and 1d). These cells also showed a remarkable susceptibility to apoptosis. When treating the 145M-*Twi* cells with exogenous psychosine for a relative short period (24h), dramatic activation of caspase-3 was observed (Fig. 3a and 3b). These findings are consistent with increased cytotoxicity of psychosine, which is significantly elevated in 145M-*Twi* cells in comparison to controls as demonstrated by the cellular LC-MS/MS assay (Fig. 2d). Similar observations were reported in earlier studies [49, 50]. Under treatment with glucosylsphingosine and psychosine, expansion of the lysosomal compartment was noted, mostly in the 145M-*Twi* cells (Fig. 3c). Given its localization in the inner mitochondrial space, cyto C staining shows a long and string pattern, reflecting intact mitochondria [51]. Under treatment with both glucosylsphingosine and psychosine, the disrupted and punctuated cyto C staining, indicating its release into the cytoplasm, was only observed in the 145M-*Twi* cells (Fig. 3c), which also demonstrated significantly higher caspase-3 activation (Fig. 3a and 3b). These results taken together explain the increased susceptibility of the 145M-*Twi* cells to apoptosis, which is in alignment with the rapid neurodegeneration observed in GLD. Since autophagy was potentially dysfunctional in LSDs [52] and also related to apoptosis, we assessed the levels of cleaved LC3B autophagy marker in comparison to controls. Under treatment with psychosine and its isomer, glucosylsphingosine, the 145M-*Twi* cells showed elevated levels of cleaved LC3B, which co-localizes with lysosomes (Fig. 4). The increased in LC3B levels can be present as part of the autophagy process, or a disruption of the autophagy flow caused by the impairment on the lysosomal system caused by the primary GALC deficiency [29]. The concentrations of psychosine and glucosylsphingosine used to treat these cells were much higher than their endogenous levels in normal and mutant cell lines. Some hydrolysis of the exogenous substrates is expected to occur. However, given the multiple pathogenic cascades secondary to a specific lysosomal enzyme, in this case GALC, it is conceivable that the 145M-*Twi* cells become more susceptible to these two cytotoxic complex lipids.

Using psychosine standards in the initial LC-MS/MS studies, we were able to determine a sensitive and consistent MRM for psychosine 462/264 (Fig. 2a). The psychosine analog, 1-acetyl-psychosine, which is absent in biological specimens, proved a reliable ISD with MRM of 504/264, and similar extraction recovery and RT (~ 0.42 min) (Fig. 2a). In MRM, the first mass analyzer is set to pass a specific precursor ion mass-charge ratio (m/z), and the second mass analyzer is set to pass a specific product ion m/z . Therefore, only ions that meet both precursor and product ion m/z conditions simultaneously will be transmitted to the detector [53]. Furthermore, because essentially all of the instrument time is spent detecting ions of interest, the detected signal intensities are greatly enhanced. The major advantage of developing a throughput assay based on LC-MS/MS is its greater sensitivity and robust throughput. The increased sensitivity of the MRM pairs for psychosine along with its short

RT (Fig. 2a) allowed the development of a practical throughput LC-MS/MS assay. This throughput assay for psychosine showed remarkable linearity for a broad concentration range (0.02–21 pmoles/well) in a 96-well plate (Fig. 6a). This linearity was also reflected in the robustness and reliability of the cellular psychosine LC-MS/MS assay performed in multi-well plates, which was confirmed by the statistical parameters generated from plate assessment (Fig. 6 and Table 1). This assessment was performed in 3 independent 96-well plates containing 145M-*Twi* and 145C-*Wt* cells and also wells with only medium (background), as per guidelines from NIH-National Chemical Genomics Center (Fig. 6) [33]. Confirming the robustness of this cellular LC-MS/MS assay, we were able to identify 145M-*Twi* cells in wells treated with the L-cycloserine (Fig. 7), which showed reduction of psychosine levels in comparison to wells containing non-treated 145M-*Twi* cells. It is important to emphasize that the establishment of these immortalized neurologically relevant cell lines were crucial to allow the development and miniaturization of the cellular psychosine LC-MS/MS assay.

5. Conclusions

The 145M-*Twi* cells showed biochemical and molecular characteristics that correlates to the GLD pathogenesis. Relevant molecular pathogenic findings involving apoptosis and autophagy were also identified in the 145M-*Twi* cells. Using a newly developed LC-MS/MS, we showed that the 145M-*Twi* cells have increased levels of psychosine, the central neuropathogenic glycosphingolipid in GLD. Given the robustness of the 145M-*Twi* cells and the LC-MS/MS assay here developed, cellular psychosine levels were measurable from cells cultured in 96-well plates. The 145M-*Twi* cells showed to recapitulate several aspects of the molecular pathogenesis of GLD and can be used as a powerful tool for the identification of therapeutic agents for this devastating neurological disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard abbreviations

Ac-DEDV-AMC	<i>N</i> -acetyl-DEVD 7-amido-4-methylcoumarin
BEH	Bridged Ethyl Hybrid
GALC	β-galactocerebrosidase
GalC	galactosylceramide

GFAP	glial fibrillary acidic protein
GLD	globoid-cell leukodystrophy
HMUβGal	6-hexadecanoylamino-4-methylumbelliferyl- β -D-galactoside
ISD	internal standard
LSD	lysosomal storage disease
MAP-2	microtubule-associated protein 2
MPA	mobile phase A
MPB	mobile phase B
MS	mass spectrometry
RT	retention time
SPE	solid-phase extraction
SV40T	SV40-transformation
<i>Tw</i>	TwitcheR

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Highlights

- A disease-cell model for globoid-cell leukodystrophy (GLD) is described.
- From brain specimens of GLD mouse model, the 145M-*Twi* cell line was established.
- The 145M-*Twi* cells revealed molecular aspects of the neuropathogenesis of GLD.
- The 145M-*Twi* cells are potential research tools for drug discovery screens.

Figure 1. Establishment and characterization of 145M-Twi and 145C-Wt cells

Primary cortical brain cells from the *Twister* (*Twi*, $GALC^{twi/twi}$) and control ($GALC^{wt/wt}$) mice were obtained from pups (day1 of life) and transformed using the SV40 large T antigen (SV40T). (a) After SV40 transformation, clones were selected from the *Twi* (145M-Twi) and control (145C-Wt). (b) The 145M-Twi cells stained for two oligodendrocyte markers: A2B5 (A and B) and galactosylceramide (GalC; D and E). Increased Galc signal were noted in 145M-Twi cells as shown in a higher magnification image in panels E (145M-Twi) and D (145C-Wt). These cells did not show MAP2 marker (panels G and H) and few cells showed astrocyte GFAP marker (J and K). (c) Restriction enzyme digestion with *EcoRV* digestion of PCR amplified fragments confirmed the homozygous mutant murine *GALC* from 145M-Twi cells (n=5), which also demonstrated reduced GALC enzymatic activity (d) in comparison to 145M-Wt controls cells (n=5).

Figure 2. Development of a LC-MS/MS method to measure psychosine

(a) Using the reverse C8 MS column in LC-MS/MS and standards for psychosine and 1-acetyl-psychosine, LC-MS/MS conditions to measure psychosine were established and respective chromatograms are shown. (b) Brain samples from infantile and late onset GLD patient were analyzed. The psychosine levels are significantly elevated in one infantile ($n=3$; $p<0.01$) and one late onset GLD brain specimens ($n=3$; $p<0.0001$), when compared to controls. (c) Chromatograms from LC-MS/MS psychosine analysis in each brain sample. (d) Lipid extracts from cell pellets were obtained from 145M-*Twi* and 145C-*Wt* ($n=3$). Psychosine levels were measured in these samples by the LC-MS/MS method here developed. The cellular psychosine levels were significantly elevated in *Twi* brain cells. (e) The LC-MS/MS chromatograms with psychosine peaks (MRM 462/264) were shown from the cell line lipid extracts. CPS, counts per second.

Figure 3. Caspase-3 activation, lysosomal compartment expansion and cytochrome C expression pattern in 145M-Twi from Twitcher

(a) Culture 145M-Twi and 145C-Wt showed baseline caspase-3 activity, key component orchestrating apoptosis. Under 24h treatment with 15 and 30 μM of glucosylsphingosine (GlucosylSP) and psychosine (PySP), the caspase-3 activation was significantly higher in the 145M-Twi cells. Triplicates were analyzed on each concentration tested. (b) Cleaved caspase-3 immunoblots (17 kD) from 145M-Twi and 145C-Wt cells treated at increasing concentrations of PySP are shown. α-tubulin (60 kD) was used as a loading control. The histogram shows the integrated density values (IDV) from the immunoblot for cleaved caspase-3. (c) Confocal immunocellfluorescence shows 145M-Twi and 145C-Wt stained with cytochrome C (Cyto C - green) and lysosomes labeled with lysosomal marker LysoTracker (LysoTr - red). Cell nuclei were stained with DAPI. The 145M-Twi and 145C-Wt were treated for 24h-period with 30 μM of glucosylsphingosine and psychosine. Increased lysosomal compartment (LysoTr -red) was noted in 145M-Twi cells. Under treatment with both glycosphingolipids, both 145M-Twi and 145C-Wt cells showed enhancement of lysosomal compartment, which was more evident in the 145M-Twi cells. Additionally, the 145M-Twi cells showed alterations in the Cyto C staining under

glucosylphingosine and psychosine treatment. The long string pattern of Cyto C, representing intact mitochondria, became punctuated and dispersed, indicating the Cyto C translocation from the inner mitochondrial space into the cytosol. Higher magnification showing the Cyto C pattern is shown on the upper right corner of each panel of treated murine 145M-*Tw*i and 145C-*W*t cells.

Figure 4. Autophagy induction marker in 145M-*Twi* cells

The 145M-*Twi* and 145C-*Wt* cells were treated for 24h-period with two different concentrations of glucosyl-sphingosine (GluSP) and psychosine (PySP). These two sphingolipids caused expansion of lysosome number and size labeled with LysoTracker Red (Lyso Tr - red). The elevated levels of cleaved LC3B (green), the autophagy inducer protein, were mostly noticeable in the 145M-*Twi* cells (arrows). The cleaved LC3B (green) showed to co-localize with the lysosomes (red) in both *Twi* and control cells (yellow signal - merge). 145M-*Twi* cells however showed the highest levels of cleaved LC-3B in both concentrations of psychosine (yellow). Higher magnification of one cell is shown on the right-hand corner of the each panel to highlight the labeled lysosomes and LC3B.

Figure 5. Specificity of LC-MS/MS assay to measure psychosine

(a) A mixture solution of psychosine (432 nM) and glucosyl-psychosine (1,080 nM) standards was prepared in methanol with 0.5% acetic acid and injected to the LC-MS/MS. The BEH Glycan column generated a doublet peak. The early peak corresponds to psychosine and the late peak to glucosylsphingosine. Psychosine and glucosyl-sphingosine are molecular isomers. (b) Lipid extracts from 145M-*Twi* cells showed a single peak corresponding to psychosine retention time (RT). (c) Lipid extract from the 145C-*Wt* showed no peak matching either psychosine or glucosyl-sphingosine.

Figure 6. Psychosine standard curves and plate assessment assays in cells cultured in 96-well plates

(a) Standard psychosine were diluted in the solvent solution (methanol:acetic acid; 99.5:0.5) at concentrations ranging from 0.02 to 21 pmoles/well. Means of measured pmoles of psychosine from 4 wells were plotted in graphs according the intensity Signals (CPS). (b–d) Plate uniformity assessment was performed using 145M-*Twi* and 145C-*Wt* cells and control (CTRL) mice cultured in three 96-well plates. 145M-*Twi* and 145C-*Wt* cells were displayed in interleaved-signal plate format including background (medium), *i.e.* alternating column-positions as three independent experiments, each in a different 96-well plate.

Figure 7. Testing throughput LC-MS/MS assay against a known psychosine-reducing agent
(a) In 96-well plates, the 145M-*Twi* and 145C-*Wt* derived from the *Twi* and respective control mice were cultured and treated for 9 days with L-cycloserine at 50 μ M, 100 μ M and 250 μ M before measuring cellular psychosine levels by LC-MS/MS. **(b)** Significant reductions of psychosine were observed in 145M-*Twi* cells with L-cycloserine in cultured medium at various concentrations (n=6). No effect of L-cycloserine was observed in the 145C-*Wt* (CTRL).

Table 1

Plate assessment for throughput LC-MS/MS cellular psychosine assay.

Plate Statistical Parameters	Mean (pmoles of psychosine/well)	SD	CV* (%)	Z' factor	Signal window
SV40-transformed cells					
Plate 1					
145M-Twi	886 × 10 ⁻³	120 × 10 ⁻³	1.38	0.57	16.03
145C-Wt	12 × 10 ⁻³	5 × 10 ⁻³	4.49	-	-
Medium (background)	7 × 10 ⁻³	14 × 10 ⁻³	19.63	-	-
Plate 2					
145M-Twi	865 × 10 ⁻³	129 × 10 ⁻³	1.52	-	-
145C-Wt	65 × 10 ⁻³	31 × 10 ⁻³	4.98	0.40	2.43
Medium (background)	44 × 10 ⁻³	46 × 10 ⁻³	10.82	-	-
Plate 3					
145M-Twi	1,053 × 10 ⁻³	125 × 10 ⁻³	1.21	0.52	3.32
145C-Wt	91 × 10 ⁻³	28 × 10 ⁻³	3.16	-	-
Medium (background)	54 × 10 ⁻³	40 × 10 ⁻³	7.67	-	-

* CV (coefficient of variation), standard deviation (SD).